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EVOLUTION OF MELANOCORTIN-2 RECEPTOR ACTIVATIONS:  
STUDIES ON A MAMMAL AND A FISH

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A Dissertation

Presented to

the Faculty of Natural Sciences and Mathematics

University of Denver

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Liang Liang

June 2013

Advisor: Dr. Robert M. Does



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Title: Evolution of Melanocortin-2 receptor activations: Studies on a mammal and a fish

Advisor: Dr. Robert M. Does

Degree Date: June 2013

## **ABSTRACT**

The structure and function relationship between melanocortin-2 receptor (MC2R) and ACTH are the most complicated in melanocortin receptor gene family. A comparative study on the activation of human and rainbow trout MC2R will provide a useful model system for understanding how ACTH emerged as the sole ligand for the MC2R of bony vertebrates. This dissertation will discuss how studies utilizing analogs of hACTH(1-24) have revealed two critical amino acid motifs in this ligand (HFRW and KKRRP) which are required for the activation of MC2R. In addition, the KKRRP motif functioned as the unique binding site for MC2R that directly contributes to the ligand selectivity feature, as revealed from studies on an ACTH antagonist which exclusively targets MC2R. Finally, based on our model for the interaction of ACTH and MC2R, the amino acid residues within TM4, EC2, and TM5 domains responsible for ACTH ligand selectivity will be evaluated by site-directed mutagenesis studies.

## **ACKNOWLEDGEMENTS**

I would like to express the deepest appreciation to my advisor, Dr. Robert Does, whose expertise, understanding, and patience, added considerably to my graduate experience. Without his guidance and persistent help, this dissertation would not have been possible. I appreciate his unsurpassed knowledge and skills in so many areas, especially his great assistant in writing.

I would like to thank the other members of my committee, Dr. Joseph Angleson, Dr. Nancy Lorenzon, and Dr. Todd Blankenship, who offered me suggestions, encouragement and support at all levels of my project. I thank Dr. George Potts for taking time out from his busy schedule to serve as my oral defense committee chair.

I also thank undergraduate student Kristin Schmid, who did excellent work on rainbow trout melanocortin-2 receptor project as her honor thesis. Her work completes this comparative study on melanocortin receptor of human and bony fish.

In addition, I would like to thank my fellow graduate students Christina Reinick, Cinda Hill, Perry Davis, and Yesenia Garcia. They each enriched my experience in the PhD program and also for all the good time in the lab over these years.

Last but not the least, I would like to thank my family and numerous friends for all of their love, patience and support that they provided me through my entire life.

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## INTRODUCTION

### **Stress and Hypothalamus-pituitary-adrenal (HPA) Axis**

The ability to evoke stress response to physiological and psychological stimuli is necessary to increase the survival rate of organisms. In mammals, common stress responses include neuroendocrine response and autonomic response, known as the two-pronged physiological defense mechanism (Pecoraro et al., 2006). The neuroendocrine stress response is regulated by hypothalamus-pituitary-adrenal (HPA) axis (Figure 1). In this circuit, stressful stimuli cause corticotropin releasing hormone (CRH) to be released from hypothalamic paraventricular nucleus (PVN) to pituitary portal circulation, and then stimulate the secretion of adrenocorticotrophic hormone (ACTH) from corticotropes in the anterior pituitary. The melanocortin peptide ACTH then induces the production of glucocorticoids in the adrenal cortex, via the activation of the ACTH receptor or the melanocortin 2 receptor (Chan et al., 2011). Consequently, glucocorticoids launch both rapid and prolonged effects on its target cells throughout the system, influencing metabolism, feeding behavior, and energy expenditure (Pecoraro et al., 2006; Mormede et al., 2011). Meanwhile, the HPA axis is under the negative feedback regulation of the circulating glucocorticoids at the level of the hippocampus, hypothalamus and pituitary (Jacobson and Sapolsky, 1991; Keller-Wood and Dallman, 1984; Cole et al., 2000).

In teleosts, steroidogenesis occurs in the interrenal tissues of the head kidney instead of adrenal gland. Therefore, the hypothalamus-pituitary-interrenal (HPI) axis mediates their neuroendocrine stress response. CRH neurons whose cell bodies are located at nucleus preopticus and nucleus lateralis tuberalis regions of the hypothalamus directly synapse to pituitary adrenocorticotrophic cells (Thomas, 2008). As the result of CRH stimulation, ACTH is released from the pituitary to the systemic circulation. Thereafter, ACTH targets the melanocortin 2 receptors (MC2R) expressed on interrenal steroidogenic cells, and activates steroidogenesis which could be sustained for several hours in some fish species (Liu et al., 2006; Mommsen et al., 1999).

### **Melanocortin Receptor Family**

Melanocortin receptors (MCRs) are G protein-coupled receptors (GPCRs), in the rhodopsin/ $\beta$  adrenergic like family A. GPCRs are single chain polypeptides forming seven membrane spanning alpha-helical regions that are linked by extracellular and intracellular domains (Devi, 2005). Conserved structural features of GPCRs found in MCRs include consensus N-linked glycosylation sites near the extracellular amino terminus and a palmitoylation site in the intracellular C-terminus (Yang, 2011; Gantz and Fong, 2003). Melanocortin receptors have short N- and C-terminus and a small second extracellular loop, therefore, they are the smallest GPCRs known. So far, five melanocortin receptors have been identified in vertebrates (Cone, 2006). Their physiological functions and ligand preference are summarized in Table 1.



**MC1R:** Human MC1R (317 amino acids) is predominantly expressed in melanocytes and melanoma cells, and it couples to the cyclic adenosine monophosphate (cAMP) signaling pathway (Chhajlani and Wikberg, 1992). Because MC1R mediates pigmentation, it is a major determinant of skin phototype and sensitivity to ultraviolet (UV) light induced skin damage (García-Borrón et al., 2005). In addition, MC1R is also found in fibroblasts, endothelial cells, monocytes, dendritic cells, neutrophils, granulocytes, osteoclasts, Leydig and lutein cells etc., suggesting its wide spectrum of physiological functions, such as anti-inflammatory and anti-pyretic actions (García-Borrón et al., 2005).

**MC2R:** MC2R (297 amino acids in humans) is also known as the ACTH receptor, since ACTH is the only ligand for this receptor (Table 1). As one of the key components of HPA/I axis, MC2R mediates the expression of steroidogenic enzymes via cAMP and PKA signaling pathway. As a result of MC2R activation, cortisol (the glucocorticoid in human and most teleosts) is secreted and elicit stress response on its target tissues. Mutations of MC2R account for approximately 25% of the cases of an autosomal recessive disease, familial glucocorticoid deficiency (FGD) (Chan et al., 2008). Other than the zona fasciculata of the adrenal cortex, MC2R is also expressed in skin (Slominski et al., 1996) and adipocytes (Boston and Cone, 1996).

**MC3R:** Human MC3R is a 361 amino acid protein, which is mainly expressed in hypothalamic and limbic regions of brain (Roselli-Reh fuss et al., 1993). *Mc3r*<sup>-/-</sup> mice showed accelerated weight gain and accumulation of extra fat mass, indicating its role in peripheral metabolism and energy balancing (Butler, 2006). Moreover, MC3R is the

only receptor in the melanocortin receptor family that can activate both cAMP, inositol triphosphate (IP<sub>3</sub>) and Ca<sup>2+</sup> signaling pathways (Konda et al., 1994; Wachira et al., 2003). Expression of MC3R is also identified in the placenta, stomach and pancreas (Gantz et al., 1993).

**MC4R:** Another receptor in this family that regulates energy homeostasis is MC4R (322 amino acids in humans). It is predominantly expressed in the brain, autonomic nervous system and spinal cord (Mountjoy and Wild, 1998). Compared to MC3R knockout mice, the obesity syndrome was far more severe in *MC4R*<sup>-/-</sup> mice, and is associated with hyperphagia, hyperinsulinemia, hyperleptinemia (Huszar et al., 1997). In addition to energy metabolism regulation, MC4R is also found to be involved in erectile dysfunction (Martin and MacIntyre, 2004) and pain (Starowicz and Przewlocka, 2003). MC4R transduces a signal by coupling to the heterotrimeric Gs protein and activating adenylate cyclase (Gantz et al., 1993).

**MC5R:** MC5R (325 amino acids) is the last melanocortin receptor to be cloned, and is expressed in skin, adrenal gland, adipose tissue, kidney, lymph nodes, liver, skeletal muscle and exocrine glands (Cooray and Clark, 2011). MC5R is involved in the production of sebaceous lipids (Chen et al., 1997), protein and tear secretion by the lacrimal gland (Entwistle et al., 1990). Activation of MC5R leads to the increase of intracellular cAMP and Ca<sup>2+</sup> (Hoogduijn et al., 2002).

## Melanocortins

The natural ligands of melanocortin receptors are melanocortin stimulating hormones (MSHs) ( $\alpha$ -MSH,  $\beta$ -MSH and  $\gamma$ -MSH) and ACTH, which are all derived from a common precursor pro-opiomelanocortin (POMC). In vertebrates, the *POMC* gene is expressed in hypothalamic neurons, anterior and intermediate pituitary, immune system and skin (Bertagna, 1994). Depending on the type of tissue, there are two outcomes in the posttranslational processing of POMC: (1) in the corticotropes of the anterior pituitary, POMC is endoproteolytically cleaved by proprotein convertase 1/3 (PC1/3). As a result, the major product is ACTH; (2) in the melanotropes of intermediate pituitary, melanocytes in skin, and certain neurons, PC1/3 and proprotein convertase 2 (PC2) work together to produce  $\alpha$ -MSH,  $\beta$ -MSH, and  $\gamma$ -MSH (Figure 2A). Other end-products cleaved from the same precursor include CLIP,  $\beta$ -lipotropin (LPH),  $\gamma$ -LPH, and  $\beta$ -endorphin (Eipper and Mains, 1980).

Although individual preference does exist, MC1R, MC3R, MC4R and MC5R can be activated by any forms of the melanocortin peptides (see Table 1). Hence, people have identified a common sequence of melanocortins known as the “HFRW” motif (shown in Figure 2B) as the binding and activation site for these receptors (Schwyer, 1977). However, “HFRW” motif is not sufficient to allow MSHs to activate MC2R, since ACTH is the only ligand for this receptor. Because  $\alpha$ -MSH [N-acetyl-ACTH(1-13)amide] is cleaved from ACTH by PC2 but unable to bind to MC2R. It appears that MC2R requires an additional binding site on the C-terminus of ACTH, which is not present in MSHs (Veo et al., 2011).

## **Melanocortin 2 Receptor Accessory Protein (MRAP)**

While the heterologous expression of MC1R, MC3R, MC4R and MC5R has been successfully achieved in mammalian cell lines, studies found that only mammalian MC2R could not be functionally expressed in heterologous cells lines unless those cell lines were derived from the adrenal cortex (Forti et al., 2006; Rached et al., 2005). In addition, it was reported that a subset of patients exhibiting FGD syndrome had functional MC2 receptors, but were resistant to ACTH treatment (Metherell et al., 2005). Collectively, these observations led to the discovery that adrenal cortical cells express melanocortin receptor accessory proteins (Metherell et al., 2005).

In the genomes of many vertebrates, there are two paralogous *mrp* genes (*mrp1* and *mrp2*) (Hinkle and Sebag, 2009; Webb and Clark, 2010). MRAPs are single chain polypeptides with a single membrane spanning domain. Both MRAP1 and MRAP2 can form antiparallel homodimers in the ER (Hinkle and Sebag, 2009), and both types of homodimers will couple to MC2R in the ER and facilitate the trafficking of mammalian MC2Rs to the plasma membrane (Figure 3). However, only MRAP1/MC2R complexes on the plasma membrane can be efficiently activated by ACTH, whereas the efficacy of ACTH activation is greatly diminished for MRAP2/MC2R complexes (Hinkle and Sebag, 2009; Webb and Clark, 2010). Furthermore, the role of MRAP1 in the trafficking and activation of MC2R is not limited to mammals, but also has been confirmed in orthologs of zebra fish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), and a frog (*Xenopus tropicalis*) (Agulleiro et al., 2010), suggesting that all vertebrate MC2Rs require MRAP1 for functional expression (Liang et al., 2011).

## Evolution of MCR and MRAP

Five melanocortin receptor coding gene were found in the genomes of tetrapods, whereas none was identified in non-chordate metazoans (Vastermark and Schioth, 2011), indicating the evolution of melanocortin receptor gene family appeared during the evolution of phylum Chordata. Thus, the evolution of this gene family would have been influenced by the two generally accepted genome duplication events (2R hypothesis) that occurred during the radiation of this phylum (Ohno et al., 1968; Lundin, 1993) and then at least one localized gene duplication event (Schioth et al., 2005). Using the evolution of HOX genes as a model (Holland et al., 1994), the generally accepted scenario (shown in Figure 4A) is that the ancestral gene of MCR undergo first duplication event and yield first two paralogous genes (MC<sub>a</sub> and MC<sub>b</sub> receptors) in agnathan vertebrates, and then the second duplication event led to four paralogous genes in the ancestral gnathostomes (Vastermark and Schioth, 2011). The fifth paralogous melanocortin receptor coding gene that is present in the genomes of teleost *Danio rerio* (Logan et al., 2003), and the tetrapods *Xenopus tropicalis* and *Mus musculus* (Cone, 2006) provides the evidence for the localized gene duplication in the early radiation of the gnathostomes (Schioth et al., 2005).

In the search for MRAP orthologs, it was noticed that the ortholog of MRAP2 but not MRAP1 is present in the genomes of sea lamprey and elephant shark, whereas Fugu (*Takifugu rubripes*) has both paralogous genes (Vastermark and Schioth, 2011). As a result, *mrp2* gene is considered as the ancestral gene of MRAP, and it appeared relatively early in the evolution of the vertebrates, but prior to the second genome

duplication event that occurred with the emergence of the gnathostomes (Ohno et al., 1968). At a later stage, the timeline of the appearance of *mrp1* gene overlaps with MC2R orthologs (Figure 4B). Phylogenetic and functional expression analysis suggests that the co-evolution of MRAP1/MC2R has diverged to yield a bony fish trend and a tetrapod trend with respect to the interaction between MC2R and MRAP1 during the radiation of the vertebrates (Liang et al., 2011).

### **Specific Aims:**

Working from these observations, this study had two objectives. The first objective was to answer the question: which regions in ACTH are responsible for the MC2R activation? We hypothesized that three zones of ACTH were involved in the MC2R activation. ACTH(1-24) consists of twenty-four residues of ACTH(1-39) that are conserved across most species and are capable of activating MC2R, and was used as the wild type ligand (Figure 2)(Costa et al., 2004). The first zone in ACTH(1-24), named Zone A for the purposes of the experiment, is where residues 6-9, HFRW, are found. This sequence appears in the sequences of  $\alpha$ - and  $\beta$ -MSH and is necessary for the activation of the other MCRs (Cone, 2006). The second region of interest consisted of residues 10-14, GKPVG (Glycine-Lysine-Proline-Valine-Glycine), and was named Zone B. The third region of interest consisted of residues 15-19, KKRRP (Lysine-Lysine-Arginine-Arginine-Proline), and was dubbed Zone C (Figure 2). The hypothesized mechanism of activation of MC2R by ACTH(1-24) is viewed as a multi-step process. The C-terminal region, Zone C, of ACTH(1-24) would make contact with MC2R at a site different from the HFRW-binding site, which would cause the opening of the HFRW-

binding site. This event would allow the HFRW motif on the ligand to bind and cause the subsequent conformational changes to the receptor and activation of the G-protein (Baron et al, 2009). The approach to validate this hypothesis was to test the activation of analogs of ACTH in Zones A, B, and C of wild type MC2R.

The second objective was to answer the question: which regions of MC2R are responsible for ACTH ligand selectivity (Figure 5)? The hypothesized model entails a docking pocket for the C-terminal residues of ACTH(1-24), Zone C, and a separate binding site for HFRW, Zone A, with a central adaptor region of ACTH, Zone B, to accommodate a better fit into the two pockets. The second docking pocket was hypothesized to be created by transmembrane regions 4 and 5 and extracellular loop 2. Individual residues in TM4, EC2, and TM5 were replaced with an alanine residue and each single mutant construct was stimulated with ACTH in a dose-dependent manner.

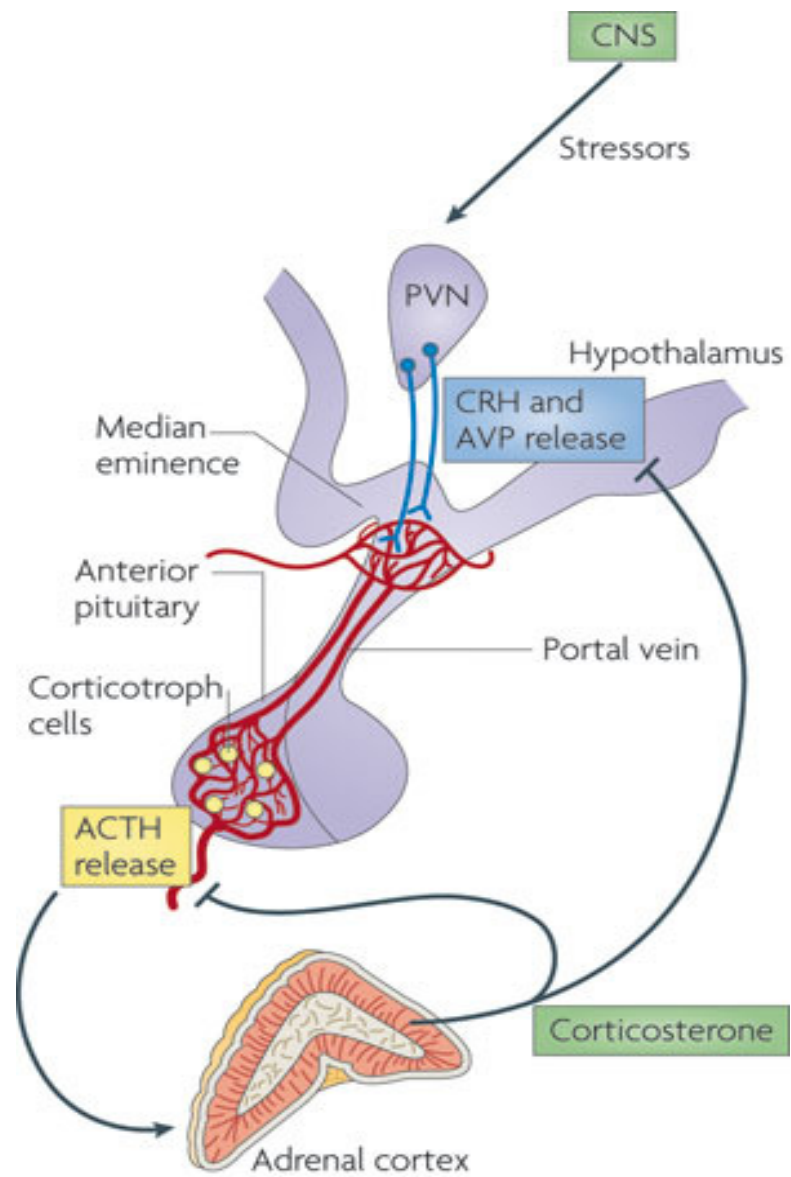


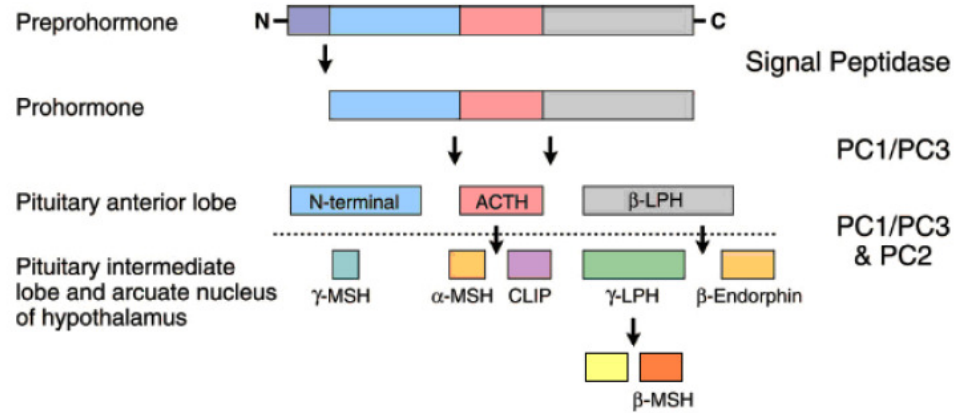
Figure 1. Hypothalamus-Pituitary-Adrenal axis in mammals (Adapted from Lightman and Conway-Campbell, 2010)



Table 1. Localization, physiological functions and ligand preference of melanocortin receptors 1 through 5 in mammals (Adapted from Cone, 2006).

Receptor	Sites of expression	Known physiological functions	Agonists
MC1-R	Melanocytes	Pigmentation (regulation of the eumelanin-pheomelanin switch)	$\alpha$ -MSH = $\beta$ -MSH = ACTH > $\gamma$ -MSH
MC2-R	Adrenal cortex	Adrenocortical steroidogenesis	ACTH
MC3-R	CNS, GI tract, kidney	Energy homeostasis, natriuresis	$\gamma$ -MSH = $\alpha$ -MSH = $\beta$ -MSH = ACTH
MC4-R	CNS	Energy homeostasis, erectile function	$\alpha$ -MSH = $\beta$ -MSH = ACTH > $\gamma$ -MSH
MC5-R	Exocrine cells	Synthesis and secretion of exocrine gland products	$\alpha$ -MSH > $\beta$ -MSH = ACTH > $\gamma$ -MSH

A.



B. [ A ][ B ][ C ]

ACTH      **SYSME****HFRW****GKPVGKKRRPVKVYPNVAENESAEAFPLEF**

α-MSH      **SYSME****HFRW****GKPV-NH<sub>2</sub>**

β-MSH    **DEGPYRME****HFRW****GSPPKD**

γ-MSH      **KYVMG****HFRW****DRF-NH<sub>2</sub>**

Figure 2. Melanocortin peptides in humans. A) Selective processing of POMC by enzymes PC1/3, and PC2. Some KR cleavage sites are suspected to be selected by PC2 B) Alignment of amino acid sequences of melanocortin peptides.

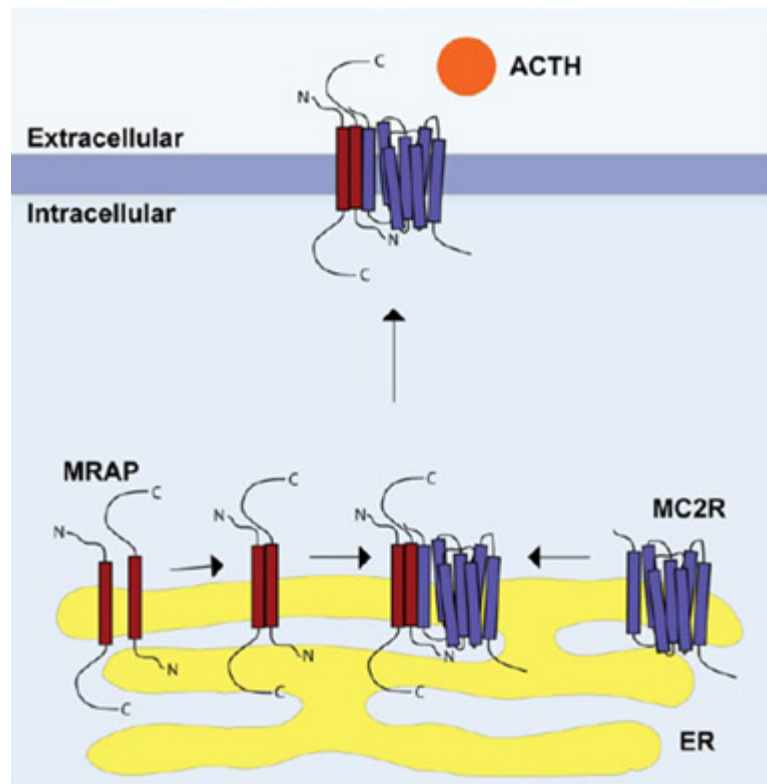
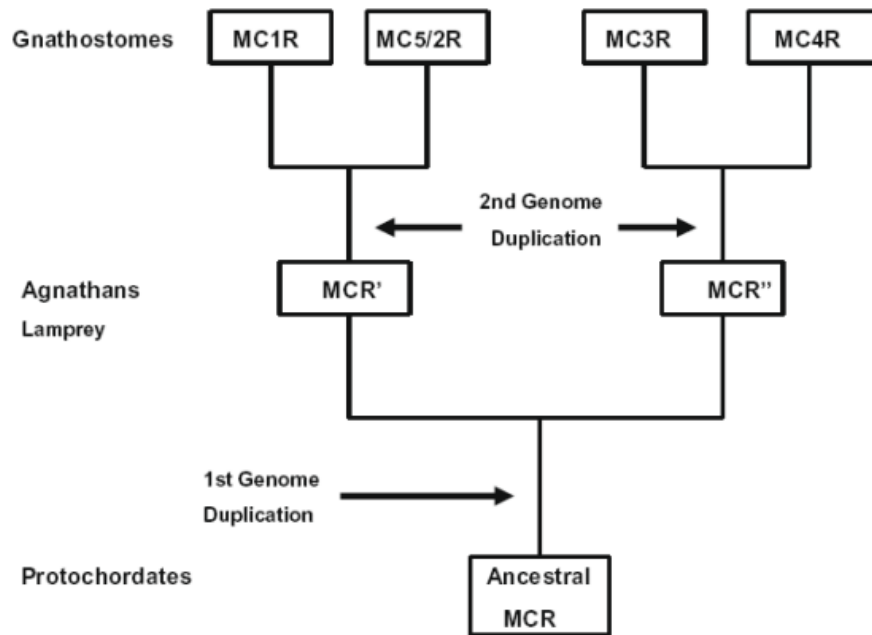


Figure 3. Melanocortin 2 Receptor Accessory Protein (MRAP) forms antiparallel homodimer in ER, and then facilitates the trafficking of MC2R to plasma membrane (Adapted from Webb and Clark, 2010).

A.



B.

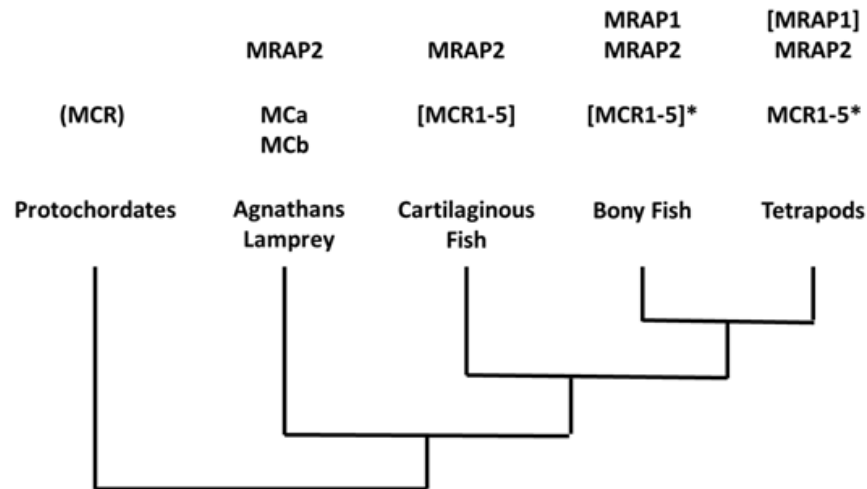


Figure 4. Modeling the evolution of melanocortin receptors in phylum chordata. A) Hypothetical scheme for the radiation of the melanocortin receptors during the evolution of the chordates. Ancestral MCR—predicted MCR gene in protochordates. MCR' and MCR''—predicted MCR genes formed after the first genome duplication event. MC5R/MC2R—predicted ancestral gene for MC5R and MC2R. B) Phylogeny of melanocortin receptors in extant vertebrates. (MCR) refers to the predicted ancestral MCR gene in protochordates. [] indicates some genes that is predicted to have been secondarily lost in some species. \* indicates MC2R in these species that can only be activated by ACTH. (Adapted from Baron et al., 2009).

## **METHODS AND MATERIALS**

### **Tissue Culture**

Chinese Hamster Ovary (CHO) cells (obtained from ATCC, VA) were used in all experiments performed. Cells were grown in Kaighn's Modification of Ham's F12K media (ATCC) supplemented with 10% fetal bovine serum, 100unit/ml penicillin, 100µg/ml streptomycin, 100µg/ml normacin, and maintained in a humidified incubator with 95% air and 5% CO<sub>2</sub> at 37°C. When reaching 80% confluence, CHO cells were split into subcultures using 0.05% trypsin/0.53mM EDTA.

### **DNA Constructs**

Human MC2R (accession no. AA067714.1) and rainbow trout MC2R (accession no. EU119870.1), were synthesized by GenScript (Piscataway, NJ) with an N-terminal V-5 epitope tag and inserted into a pcDNA3.1+ vector. Mouse (*Mus musculus*) MRAP1 (accession no. NM\_029844), zebrafish (*Danio rerio*) MRAP1 (accession no. XR\_117835), were individually synthesized by GenScript with an N-terminal FLAG epitope tag and were separately inserted into pcDNA3.1+ vectors. cAMP reporter construct CRE-Luc (Chepurny and Holz, 2007) was provided by Dr. Patricia Hinkle (University of Rochester, NY). All DNA constructs were inserted in pcDNA3.1+ vector individually.

Mutations were introduced to receptor constructs by site-directed mutagenesis with individual alanine substitutions (Performed by GenScript, Piscataway, NJ). Positions in hMCc2R and rtMC2R examined by this study were shown in Figure 5.

### **ACTH Analog Peptides**

Human adrenocorticotrophic hormone ACTH(1-24) and NDP- $\alpha$  MSH was purchased from Sigma-Aldrich Inc.(St. Louis, MO). The rest of hACTH(1-24) analogs including alanine substitutions and truncated peptides were synthesized by New England Peptide Inc. (Boston, MA). The amino acid sequences of hACTH(1-24) analogs used in this study are listed in Table 2.

### **Immunocytochemistry**

CHO cells were seeded in 8-well chamber slides ( $2.5 \times 10^4$  cells/well), and after 24 hours, transfected with 1  $\mu$ g of DNA construct using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and OptiMEM (Mediatech Inc, Herndon, VA). Cells were allowed to express the receptors/MRAPs for 24 hours and fixed with 4% PFA for 15 minutes, washed, and then half of the wells were permeablized with 0.3% triton X-100 for 10 minutes. Primary antibodies (mouse anti V5 and/or rabbit anti FLAG, 1:500 dilution) were applied to the cells for 1 hour at 37°C. After three washes with PBS, cells were incubated with secondary antibodies (donkey anti mouse conjugated with Alexa388 and/or donkey anti rabbit conjugated with Alexa555, 1:800 dilution) for 45 minutes at 37°C. After three washes with PBS, chambers were removed from slides. Coverslips

were mounted with Vecta-shield (Vector Laboratories Inc, Burlingame, CA), and nuclei were stained with DAPI (blue). Slides were imaged using 100X oil immersion objective with a fluorescent Zeiss Axioplan 2 microscope equipped with Hamamatsu digital camera. All images were analyzed with SlideBook ([www.slidebook.com](http://www.slidebook.com)) software.

### **cAMP Reporter Assay**

For the cAMP assay,  $2.5 \times 10^6$  CHO cells were cotransfected with MC2R, MRAP, and CRE-Luc (Chepurny and Holz, 2007) DNA constructs (2 $\mu$ g each) using Amaxa Cell Line Nucleofector II system (Lonza, MD) with solution T and program U-23. After 10-minute recovery, cells were then seeded in white 96-well plate with a final density of  $1 \times 10^5$  cells/well. 48 hours after transfection, cells were stimulated with ACTH analogs diluted within serum-free CHO media for 4 hours. Analogs were tested at doses ranging from  $10^{-6}$  M to  $10^{-14}$  M, and each dose was tested in triplicate against a wild type ACTH(1-24) control. Then stimulating media were removed, Luciferase substrate reagent Bright GLO (Promega, WI) was applied to the wells, and incubated at room temperature for 5 mins. Luminescence was measured by Bio-Tek Synergy HT plate reader (Winooski, VT). To determine the basal levels of cAMP production, transfected CHO cells stimulated with vehicle were measured along with each experiment group individually. Luminescence readings were first subtracted by their basal activity and then fit to Michaelis-Menton equation to obtain EC<sub>50</sub> values. Data were analyzed using Kaleidograph software ([www.synergy.com](http://www.synergy.com))

### **cAMP Enzyme Immunoassay**

Cells transiently transfected with MC2R and MRAP (Amaxa nucleofection kit T) were plated in 6-well plates at a density of  $3 \times 10^5$  cells/well and allowed to recover from transfection for 48 hours. ACTH analogs were diluted as needed in standard extracellular solution (10mM D-glucose, 2mM KCl, 1mM  $MgCl_2$ , 5mM  $CaCl_2$ , 140mM NaCl, 10mM HEPES and 0.5% BSA). 1mM 3-Isobutyl-1-methylxanthine (IBMX) was used in solutions to prevent the degradation of intracellular cAMP from phosphodiesterase. Analogs were tested at concentrations ranging from  $10^{-6}M$  to  $10^{-10}M$ , and each dose was tested in triplicate against a wild type ACTH(1-24) control. After 15-minute incubation at  $37^\circ C$ , analogs were removed from the wells, and the cells were lysed and collected within 0.1M HCl after well scraping. Lysate was centrifuged at 4.5RCF for 5 minutes, and the supernatant containing cAMP was assayed immediately using a direct cAMP EIA kit (Assay Design, Ann Arbor, MI) and measured with Bio-tek Syngerty HT plate reader (Winooski, VT).

### **Radioligand Binding Assay**

CHO Cells transiently transfected with MC2R and MRAP (Amaxa nucleofection kit T) were plated in 12-well plates at a density of  $2 \times 10^5$  cells/well. Assay was performed after for 48 hours after transfection for cell recovery and receptor expression. To stabilize these cells, binding buffer (10mM Glucose, 1mM  $MgCl_2$ , 10mMHEPS, 5mM  $CaCl_2$ , 140mM NaCl, 2mM KCl, with 0.1% BSA) was applied to each well, incubated for 20 min at  $37^\circ C$  and then removed.  $1 \times 10^5$  cpm ACTH(1-39)Tyr23I<sup>125</sup>(Perkin Elmer,



Waltham, MA) was used in combination of non-radiolabeled ACTH analogs within binding buffer. After 1 hour incubation at 37°C, binding reaction was terminated by removing the binding buffer and followed by three washes with cold PBS on ice. Cells were then solubilized with 0.1% SDS for 30 minutes at room temperature. After well scraped, cell lysate was collected and the radioactivity was quantified by a gamma counter.

### **Cell Surface ELISA**

CHO cells were seeded in 24-well plates (5×10<sup>4</sup> cells/well), and after 24 hours, transfected with 250ng of each DNA construct using lipofectamine 2000 (Invitrogen, CA). Cells were allowed to express the receptors for 24 hours and fixed with 4% PFA. 0.3% triton X-100 was applied to half of the wells to permeablize the cells. Then blocking buffer (5% dry milk in PBS supplemented with 10% FBS and 5% normal goat serum) was applied to the well overnight at 4°C. cells were incubated with primary antibodies mouse anti V5 and/or rabbit anti FLAG(1:2000) for 1 hour at 37°C, washed three times for 5 minutes, followed with secondary antibody goat anti-mouse conjugated with HRP (1:2000) for 45mins at 37°C. Wells were again washed three time for 5 minutes, and allowed to dry before adding 200 µL TMB (Invitrogen, MD) and allowed to incubate for 10 minutes at room temperature. The reaction was halted by adding 200 µL 1N HCl and gently swirling. 300 µL from each well was transferred to a 96-well plate and read immediately at absorption 450nm by Bio-Tek Synergy HT plate reader (Winooski, VT). All experiments were performed in triplicates with a GFP transfected

control. Data were normalized to GFP control and analyzed using Student's t-test for equal variance with Kaleidograph software ([www.synergy.com](http://www.synergy.com)).

### **Statistical Analysis**

Data points are expressed as the mean with standard error of values obtained from experiments which were performed in triplicates. Differences between experimental treatments with corresponding controls were determined using unpaired two-tailed Student's t-test for equal variance. Significance was set at  $P \leq 0.05$ .

[illegible]

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Table 2. hACTH(1-24) analogs used in this study. Alanine substitutions in amino acid sequences are underlined.

ACTH Analogs		Amino Acid Sequences
Wild Type	ACTH (1–24)	SYSMEHFRWGKPVGKKRRPVKVYP
Zone A	A4/HFRW	SYSME <u>AAAA</u> GKPVGKKRRPVKVYP
	HFAW	SYSMEHF <u>A</u> WGKPVGKKRRPVKVYP
	HARW	SYSMEH <u>A</u> RWGKPVGKKRRPVKVYP
	AFRW	SYSME <u>A</u> FRWGKPVGKKRRPVKVYP
	HFRA	SYSMEHFRA <u>G</u> KPVGKKRRPVKVYP
Zone B	A10–14	SYSMEHFRW <u>AAAAA</u> KRRPVKVYP
	A10/14	SYSMEHFRWAKPVAKRRPVKVYP
	KP	SYSMEHFRWG <u>AA</u> VGKKRRPVKVYP
	P12	SYSMEHFRWGKA <u>V</u> GKKRRPVKVYP
	ACTH (1–22)	SYSMEHFRW–KPV–KKRRPVKVYP
	ACTH (1–21)	SYSMEHFRWG---GKKRRPVKVYP
Zone C	A5/Tetra	SYSMEHFRWGKPVGA <u>AAAAA</u> VKVYP
	AARRP	SYSMEHFRWGKPVGA <u>AARRP</u> VKVYP
	KKAAA	SYSMEHFRWGKPVGKK <u>AAA</u> VKVYP
	AAW	SYSMEHFRWGKPVGKRA <u>AAW</u> VKVYP
Zone D	A20–23	SYSMEHFRWGKPVGKKRRP <u>AAAA</u> P
	V20	SYSMEHFRWGKPVGKKRRP <u>A</u> KVYP
	K21	SYSMEHFRWGKPVGKKRRPV <u>A</u> VYP
	V22	SYSMEHFRWGKPVGKKRRPVKA <u>P</u>
	Y23	SYSMEHFRWGKPVGKKRRPVKV <u>A</u> P
	P24	SYSMEHFRWGKPVGKKRRPVKVYA <u></u>
$\alpha$ -MSH Analog	NDP- $\alpha$ MSH ACTH (4–10)	Ac-SYSMEHFRWGKPV MEHFRWG
C-terminal Analog	KPV	KPVGKKRRPVKVYP
	Ac-GKKRRP	Ac-GKKRRPVKVYP
	Ac-KKRRP	Ac-KKRRPVKVYP
	KKRRP	KKRRPVKVYP
	KKRRPAs	KKRRP <u>AAAAA</u>
	RRP	RRPVKVYP
	PVK	PVKVYP

## **CHAPTER ONE**

### **Ligand Selectivity Properties of Human Melanocortin-2 Receptor**

The ligand selectivity feature makes melanocortin 2 receptor a very special member in its gene family. Unlike other melanocortin receptors, mammalian MC2Rs, and perhaps all bony vertebrate MC2Rs, can only be activated by ACTH, but not by any MSHs (Cone, 2006). This chapter will focus on identifying the critical amino acid motifs that are responsible for hMC2R ligand selectivity by analyzing various regions of hACTH sequence.

As noted in the Introduction, the full length of ACTH peptide in most species of vertebrates is 39 amino acid residues (Dores and Lecaude, 2005). However, earlier studies reviewed by Schwyzer (1977) indicated that the functional domain required for activating the adrenal “ACTH” receptor was located within the first 24 residues of this hormone. Hence, in this study, hACTH(1-24) was used in all the experiments representing mammalian adrenocorticotrophic hormone. As summarized by Schwyzer (1977), truncated analog studies have uncovered two functional motifs within ACTH: the HFRW motif and the KKRR motif, which will be reevaluated in this study using alanine substitution analogs in order to retain the secondary structure of ACTH as much as possible.

Since the HFRW motif is present in all of the melanocortin peptides (Nakanishi et al., 1979), the key to understanding the ligand selectivity of an MC2R would appear to lie in residues 14 to 24 in the sequence of ACTH(1-24). As a result, the importance of the C-terminal domain will be carefully examined utilizing alanine substitution analogs of hACTH(1-24). Based on the sequence of interest, ACTH analogs examined in this chapter were grouped into four categories (Table 2): (1) Zone A analogs—H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup>; (2) Zone B analogs—G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup>; (3) Zone C analogs—K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup>P<sup>19</sup>; (4) Zone D analogs—V<sup>20</sup>K<sup>21</sup>V<sup>22</sup>Y<sup>23</sup>P<sup>24</sup>.

## **Results**

### **Ligand Selectivity of hMC2R**

#### ***Zone A Analogs***

To determine the importance of each amino acid residue in HFRW motif, we designed and examined five Zone A analogs (See sequences in table 2) on CHO cells transiently transfected with hMC2R and mMRAP1 (Figure 6A). Consistent with previous studies, A4 analog which has four alanine substitutions at HFRW motif cannot stimulate the hMC2R activation. In order to test how these positions of this region contribute to the activation of receptor, analog AFRW, HARW, HRAW, and HFRA was applied individually to stimulate the transfected cells. Analog HFRA was unable to activate hMC2R in the same manner of A4 analog, indicating that the tryptophan residue plays an essential role in this motif. Moreover, although analog AFRW, HARW, and HRAW

were able to activate hMC2R, the shifts in EC<sub>50</sub> values of their activation curves were 8-fold, 8000-fold, and 440-fold as compared to ACTH(1-24) (Table 3). Student t-test analysis showed that alanine substitution on phenylalanine (HARW) and arginine (HFAW) significantly diminished receptor activation. As a result, the order of the importance of these positions is W<sup>9</sup>>F<sup>7</sup>>R<sup>8</sup>>H<sup>6</sup>.

### ***Zone B Analogs***

The region between HFRW and KKRRP was also first examined using four ACTH(1-24) analogs with alanine substitutions at G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup>, G<sup>10</sup>G<sup>14</sup>, K<sup>11</sup>P<sup>12</sup>, and P<sup>12</sup> (Analog A10-14, AKPVA, GAAVG, and GKAVG in Table 2) on transfected CHO cells. Analog A10-14 showed significantly reduced activation activity with a 900-fold increase in the EC<sub>50</sub> value, whereas other analogs substituted with single or double alanines in this region did not show any obvious decline as compared to ACTH(1-24) (Figure 6B and Table 3). Nevertheless, when we tested two truncated analogs ACTH(1-21) and ACTH(1-22) that skip position G<sup>10</sup>G<sup>14</sup> or K<sup>11</sup>P<sup>12</sup>V<sup>13</sup> within this domain, ACTH(1-21) completely lost the stimulatory effect to hMC2R while the ACTH(1-22) response was undetectable until 10<sup>-7</sup>M (Figure 6C).

### ***Zone C Analogs***

Three ACTH analogs with alanine substitutions (See sequences in Table 2) were synthesized in order to evaluate the importance of KKRRP motif in terms of hMC2R activation on transfected CHO cells. Replacing KKRRP with five alanines (A5 analog)

greatly reduced the activation of receptor by 3000-fold shifting in  $EC_{50}$  (Figure 6D and Table 3). Further, position  $K^{15}K^{16}$  and  $R^{17}R^{18}P^{19}$  were tested separately using analog AARRP and KKAAA. The  $EC_{50}$  values of both analogs were significantly increased as compared to ACTH(1-24), while analog KKAAA is a less potent ligand compare with AARRP (Table 3).

### ***Zone D Analogs***

Albeit alanine substitution at KKRRP motif introduced a significant right shift in ACTH dose response curve, note that it was still able to stimulate a weak response at concentration of  $10^{-7}M$  (Figure 6B). This observation raise the possibility that the subsequent sequence  $V^{20}K^{21}V^{22}Y^{23}P^{24}$  may play a role in facilitating ACTH(1-24) binding to the receptor. To test this hypothesis, six Zone D analogs (A20-23, V20, K21, V22, Y23 and P24) were examined on hMC2R (see sequences in Table 2), whereas all of these analogs did not affect the activation curve as compared to ACTH(1-24) (Figure 7).

### **Binding Affinity Analysis**

In addition to receptor activation assay, analogs from each region that showed great reduction in activation activities were further examined with radioligand binding assay (Figure 8A). A right shift in binding curve showed in all three analogs (A4, A5 and A10-14), while only the  $K_i$  value of analog A5 was significantly greater as compared to ACTH(1-24) ( $P<0.05$ ).



## Discussion

The HFRW motif is present in all of the melanocortins (Nakanishi et al., 1979). N-terminally truncated analogs of mammalian ACTH(1-24) in which this region of the polypeptide has been eliminated lack the ability to activate any melanocortin receptor (Eberle and Schwyzer, 1975; Schwyzer, 1977). Hence, the HFRW motif has been identified as the core sequence of all melanocortin peptides, and consistently in this study, alanine substitutions in this motif (A4 analog) completely abolished the activation of hMC2R as indicated in Figure 6A. However, not all four positions in this motif are equally important for the activation of hMC2R. It appears that tryptophan<sup>9</sup> plays a prominent role in the functioning of this motif, since alanine substitution on this position caused a complete loss in activation as analog A4 did. Assuming that the HFRW motif in ACTH(1-24) also forms a reverse  $\beta$ -turn as observed for  $\alpha$ -MSH (Ying et al., 2003), the backbone structure held by the stacking of histidine<sup>6</sup> and phenylalanine<sup>7</sup> aromatic rings would still be intact in analog HFRA. Thus, the side chain of tryptophan<sup>9</sup> was probably interacting with certain sites of hMC2R which is critical for activation. Other than tryptophan<sup>9</sup> and phenylalanine<sup>7</sup> which is the central residue in the reverse  $\beta$ -turn, alanine substitution at arginine<sup>8</sup> greatly affected receptor activation as well. As reported by Lee and colleagues (1998), the same arginine<sup>8</sup> in  $\alpha$ -MSH analog was responsible for the electrostatic interactions between the ligand and the complementary residues of the receptors (e.g., the TM3 residue D122 in hMC4R). When Zone A analogs were applied to human MC4R, similar effects showed in the activation curves (Figure 8B). The activation

of hMC4R required the presence of tryptophan<sup>9</sup>, which appears to be the most significant residue in HFRW motif as well. However, every residue in this motif was important for hMC4R activation, and the order of importance of other residues was: arginine<sup>8</sup>>phenylalanine<sup>7</sup>>histidine<sup>6</sup> (Table 4). Based on these observations, it is reasonable to postulate that the HFRW motif is an important binding site for hMC2R, but at least in terms of activation it is slightly different with the mode of hMC4R. According to Pogozheva et al. (2005), of the twelve residues in TM2, TM3, TM6 and TM7 of hMC4R that are involved in the binding of HFRW motif, ten amino acids are conserved residues in melanocortin receptors (Figure 9). Six amino acids are identical in all five melanocortin receptors, while four amino acids (F129 in TM3, V262 and V265 in TM6, and G288 in TM7) are unique in MC2R. The differences in residues may lead to a boarder HFRW binding site for MC4R, hence, alanine substitution at HFRW motif had a greater effect on MC2R activation as compared to MC2R.

As reviewed by Schwyzer (1977), the shortest analog of ACTH(1-24) that still retains some level of biological activity is ACTH(1-16), and conversely analogs longer than ACTH(1-16) are progressively stronger agonists. Costa et al. (2004) conducted a series of studies that focused on residues in the motif KKRRP in the sequence of hACTH(1-24). Alanine substitutions at any of these sites lowered the biological activity of the ACTH(1-24) analog with varying degrees of efficacy. Collectively, the experiments using C-terminally modified analogs of ACTH(1-24) point to the KKRRP motif in mammalian ACTH(1-24) as an important region for the activation of MC2R. In agreement with this hypothesis, the replacement of the KKRRP motif in hACTH(1-24)

with alanines (A5 analog) produced an analog with a greatly diminished capacity for activating MC2R (Figure 6B). This reduction in activation was probably resulting from the significant drop of ligand binding affinity (Figure 8A). With a closer look in KKRRP motif, the R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> motif appears to be more critical than the K<sup>15</sup>K<sup>16</sup> motif (Figure 6B and Table 3). Although analog A5 introduced a right shift in activation curve, it was still able to bind to and stimulate hMC2R at concentrations higher than 10<sup>-7</sup>M (Figure 6B), implying the involvement of the subsequent sequence VKVYP at C-terminal of ACTH(1-24). Albeit alanine substitution at this domain didn't affect hMC2R activation curves (Figure 7), truncated analog KKRRP partially lost its competitive ability as compared to KKRRP analog (See Chapter Two Figure 12B), probably due to a reduction of binding affinity. Taken together, these observations supported the hypothesis that KKRRP motif functions as a secondary binding site of ACTH for the activation of hMC2R.

Accepting the premise that there is an HFRW binding site as well as a KKRRP binding site on hMC2R, we next wished to address the question of the relative distance between these two binding sites. As indicated in Figure 7, substitution with five alanines at this region greatly reduced activation activities of hMC2R, while the truncated Zone B analog, neither ACTH(1-21) nor ACTH(1-22), was able to stimulate this receptor. Based on these observations, it seems that the G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup> sequence, especially the distance between HFRW and KKRRP motif, would play a critical role in positioning the ligand into the corresponding binding sites of these two motifs. Assuming that initial contact with the hMC2R occurs at the proposed KKRRP binding site, and this binding event would then lead to the docking of the HFRW domain into its corresponding binding

site of the receptor, altering the secondary structure of the GKPVG region of hACTH(1-24) would cause the HFRW motif unable to be properly positioned into the HFRW binding site on the receptor (Figure 10). Based on these assumptions, this model would explain the inability of  $\alpha$ -MSH to activate MC2R, or even to act as a competitive inhibitor of ACTH(1-24) (Buckley et al., 1981). It would appear that since  $\alpha$ -MSH lacks the KKRRP motif, this ligand cannot be properly oriented for inserting into the HFRW binding site in melanocortin-2 receptor. On the other hand,  $\alpha$ -MSH can successfully activate MC1R, MC3R, MC4R, and MC5R, even without a complete KKRRP sequence (Cone, 2006). In this regard, it would be reasonable to propose that these MCRs may have a flexible binding site for HFRW motif, which allow both  $\alpha$ -MSH and ACTH(1-24) to activate MC1R, MC3R, MC4R, and MC5R.

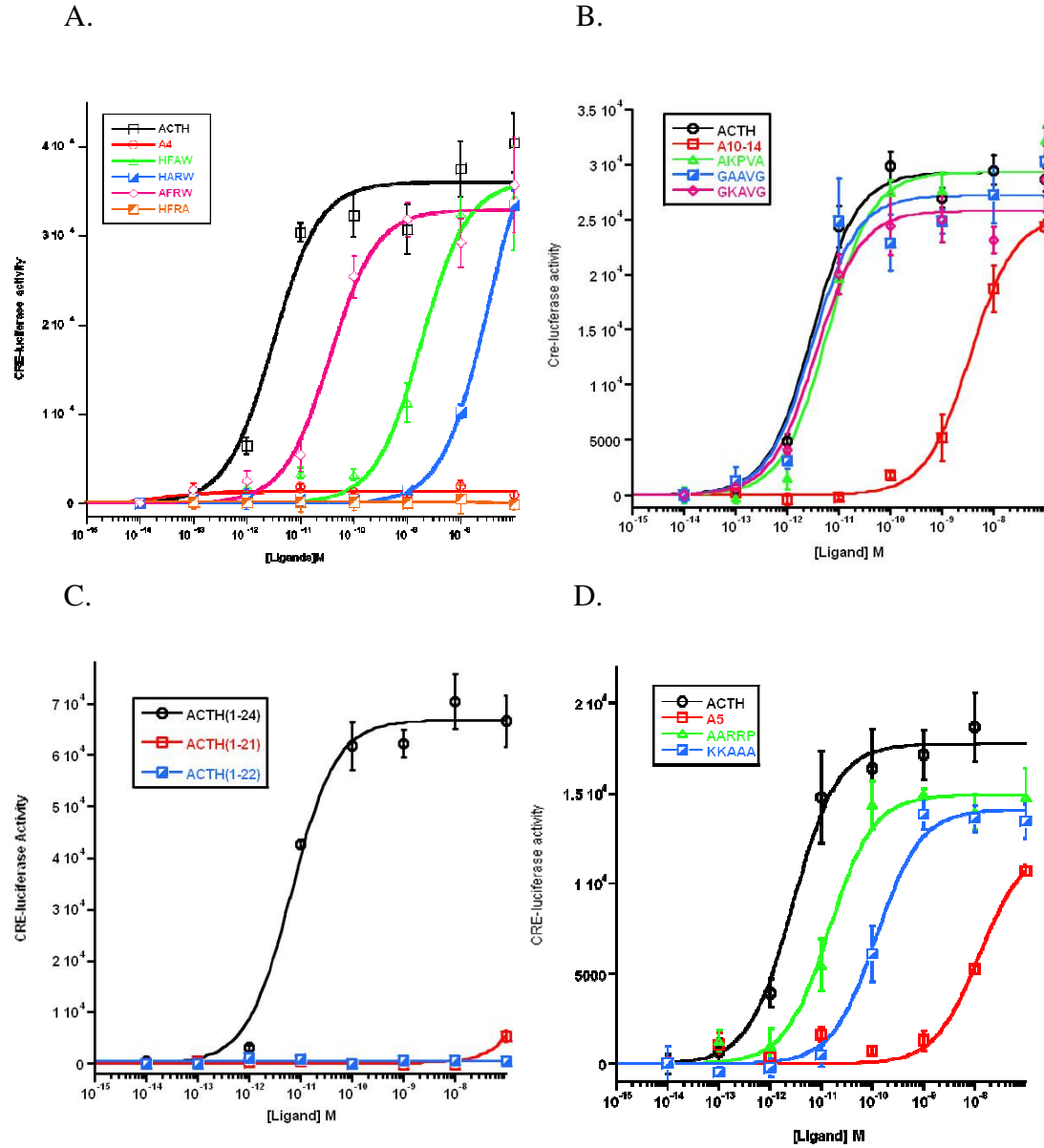


Figure 6. Activation curves of hMC2R by the stimulation of hACTH(1-24) analogs: A) Zone A analogs; B) Zone B alanine substitution analogs; C). Zone B truncated analogs; D) Zone C analogs

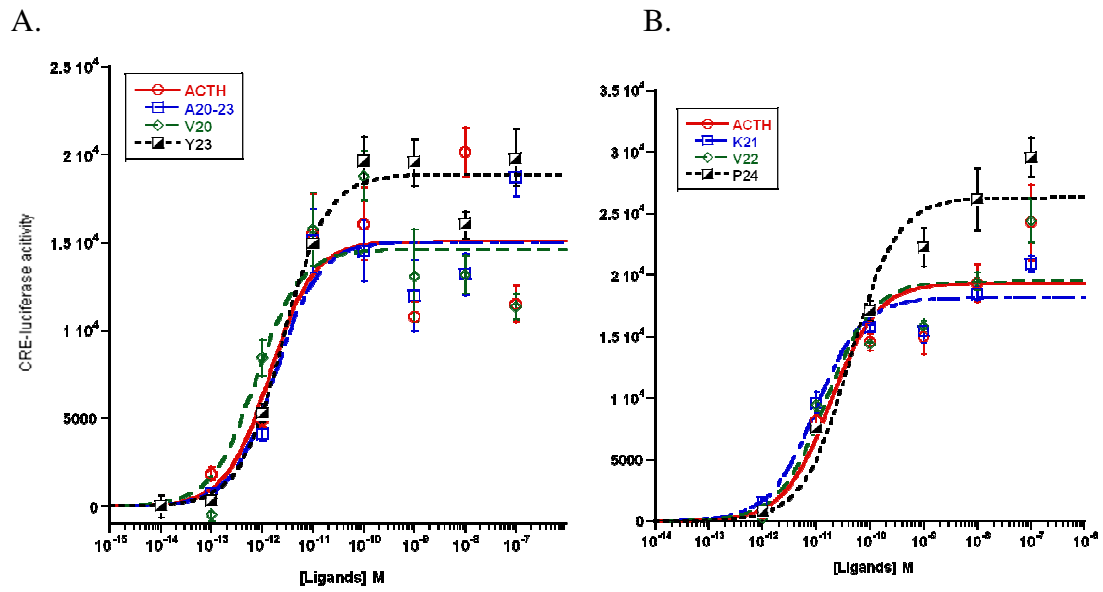


Figure 7. Activation curves of hMC2R by the stimulation of hACTH(1-24) Zone D analogs.

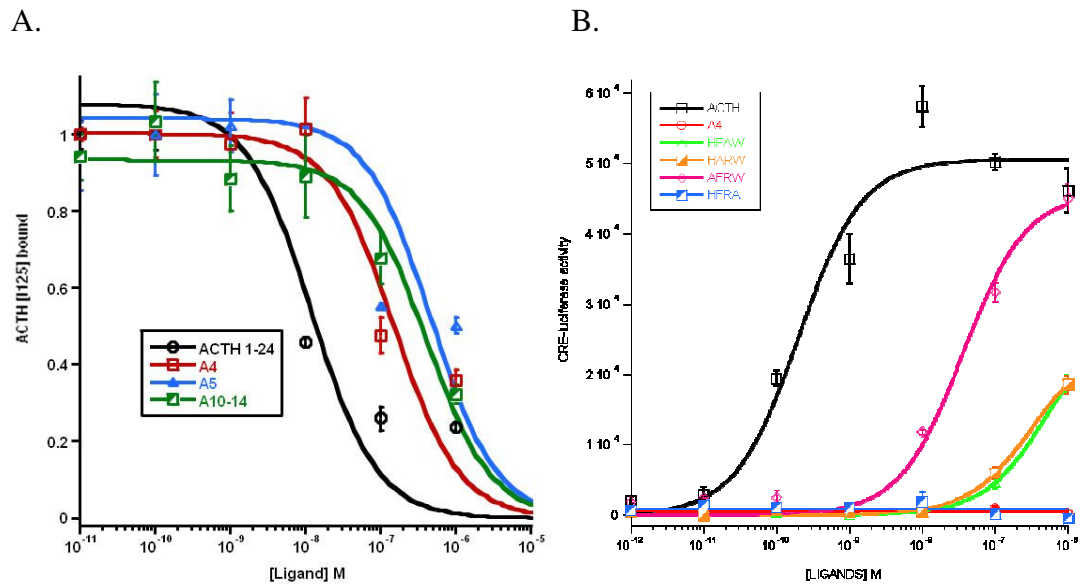


Figure 8. A) hMC2R ligand binding analysis. B) Activation curves of hMC4R by the stimulation of hACTH(1-24) Zone A analogs.

Table 3. Effects of ACTH(1-24) analogs on hMC2R activation and <sup>125</sup>I-ACTH binding

ACTH Analogs		EC <sub>50</sub> ± SEM (pM)	P Values	K <sub>i</sub> ± SEM (nM)	P Values
Zone A	ACTH(1-24)	4.2 ± 1.3		11.8 ± 8.4	
	A4	NA		148.2 ± 79.8	0.08
	AFRW	36 ± 11	0.057		
	HARW	33505 ± 2559***	0.0001		
	HFAW	1845 ± 467**	0.008		
	HFRA	NA			
Zone B	A10-14	3608 ± 381***	0.0004	396.6 ± 126.7	0.2
	AKPVA	6.1 ± 1.7	0.2		
	GAAVG	3.1 ± 1.4	0.7		
	GKAVG	3.7 ± 1.1	0.6		
	ACTH(1-22)	NA			
	ACTH(1-21)	NA			
Zone C	A5	12214 ± 4745*	0.03	459.7 ± 466.1*	0.02
	AARRP	14.4 ± 3.3*	0.02		
	KKAAA	120 ± 24.5**	0.004		

EC<sub>50</sub> values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (\*:P<0.05, \*\*:P<0.005, \*\*\*: P<0.005). NA, No activation



Table 4. Effects of ACTH(1-24) Zone A analogs on hMC4R activation

ACTH Analogs	EC <sub>50</sub> ± SEM (nM)	P Values
ACTH(1-24)	0.2 ± 0.85	
A4	NA	
AFRW	36 ± 8.9*	0.008
HARW	3128 ± 424**	0.0009
HFAW	5284 ± 580***	0.0004
HFRA	NA	

EC<sub>50</sub> values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (\*:P<0.05, \*\*:P<0.005, \*\*\*: P<0.005). NA, No activation

```

                                N-terminal                                [-----TM1-----]
hMC2R MK-----HIINSYENINNTARNNSDCPRVVLPEEIFFFTISIVGVLENLIVL
hMC4R MVNS----THRGMHTSLHLWNRS---SYRLHSNASESLGKGYSDGGCYEQLFVSPFVFTLGVISLLENILVI
hMC1R M-----AVQGSQRRLLGSLNST-----PTAIPQLGLAANQTGARCLEVISISDGLFLSLGLVSLVENALVV
hMC3R MNAS-----CCLPSVQPTLPNGS-----EHLQAPFFSNQSSSAFCEQVFIKPEVFLSLGIVSLLENILVI
hMC5R MNSS-----FHLHFLDLNLNAT-----EGNLS---GPNVKNKSSPCEDMGIAVEVFLTLGVISLLENILVI

--]      IC1      [-----TM2-----]      EC1      [-----TM3-----]
hMC2R LAVFKKNLQAPMYFFICSLAISDMLGSLYKILENIIILRNMGYLKPRGSFETTADDIIDSFLVLSLLGSI
hMC4R VAIKKNLHSPMYFFICSLAVADMLVSVSNGSETIV-ITLLNSTD-TDAQSFTVNIDNVIDSVICSSLLASI
hMC1R ATIAKNRNLHSPMYCFICCLALSDLLVSGSNVLETAV-ILLLEAGALVARAAVLQQLDNVIDVITCSSMLSSL
hMC3R LAVVRNGLHSPMYFFICSLAVADMLVSVSNALETIM-IAIVHSDYITFEDQFIQHMDNIFDSMICISLVASI
hMC5R GAIKKNLHSPMYFFVCSLAVADMLVSMSSAWETIT-IYLLNNKHLVIADAFVRHIDNVFDSMICISVVASM
                                *      *      *      *

----]      IC2      [-----TM4-----]      EC2      [-----TM5-----]
hMC2R FSLSVIAADRYITIFHALRYHSIVTMRRTVVVLTVIWTFCTGTGITMVIFSHHVPTVITFTSLFPLMLVFLC
hMC4R CSLLSIAVDRYTIFIALRYHSIVTLPRARRAVAAIWVASVVFSTLFIAYYDHVAVLLCLVVFFLAMLVLMVAV
hMC1R CFLGAIADRYISIFIALRYHSIVTLPRARRAVAAIWVASVVFSTLFIAYYDHVAVLLCLVVFFLAMLVLMVAV
hMC3R CNLLAIAVDRYVTIFIALRYHSIMTVRKALTLIVAIWCCGVCVGVFIVYSESKMVIVCLITMFFAMMLLMGT
hMC5R CSLLAIAVDRYVTIFIALRYHHIMTARRSGAIIAGIWAFCCTGCGIVFITYSESTYVILCLISMFFAMFLFLLVS

-----]      IC3      [-----TM6-----]      EC3
hMC2R LYVHMFLLARSHTRKISTLP-----RANMKGAITLTILLGVFIFCWAPFVLHVLLMTFCPSNPYCA
hMC4R LYVHMFLLMARLHIKRIAVLP-----TGAIROGANMKGAITLTILLGVFVVCWAPFFLHLIFYISCPQNPYCV
hMC1R LYVHMLARACQHAQGIARLHK-----RQRPVHQGFGLKGAVTLTILLGIFFLCWGPFFLHLTLIVLCPEHPTCG
hMC3R LYVHMFLLARLHVKRIAALP-----PADGVAPQOHSCMKGAVTITILLGVFIFCWAPFFLHLVLIITCPTNPYCI
hMC5R LYIHMFLIARLHVKRIAALPGASS-----ARQRTSMQGAIVTMTLLGVFTVCWAPFFLHLTLMLSCPONLYCS
                                *      *      *      *

[-----TM7-----]      C-terminal
hMC2R CYMSLFQVNGMLIMCNAVIDPFIYAFRSPELRDAFKKMIFCSRYW
hMC4R CFMSHFNLYLILIMCNSIIDPLIYALRSQELRKTFKEI-ICCYPLGGLCDLSSRY
hMC1R CIFKNFNLFLALIICNAIIDPLIYAFHSQELRRTLKEVLTCSW
hMC3R CYTAHFNTYLVILIMCNSVIDPLIYAFRSLELRNTFREILCGCNGMNLG
hMC5R RFMSHFNMYLILIMCNSVMDPLIYAFRSQEMRKTFKEI-ICCRGFRIACSFPRRD
                                *      *

```

Figure 9. Human MCRs sequence alignment. Shaded residues indicate sites in which at least 60% of the amino acids at that position are identical for all sequence. \*: Positions involved in the binding of HFRW motif in MC4R (Pogozheva et al., 2005).

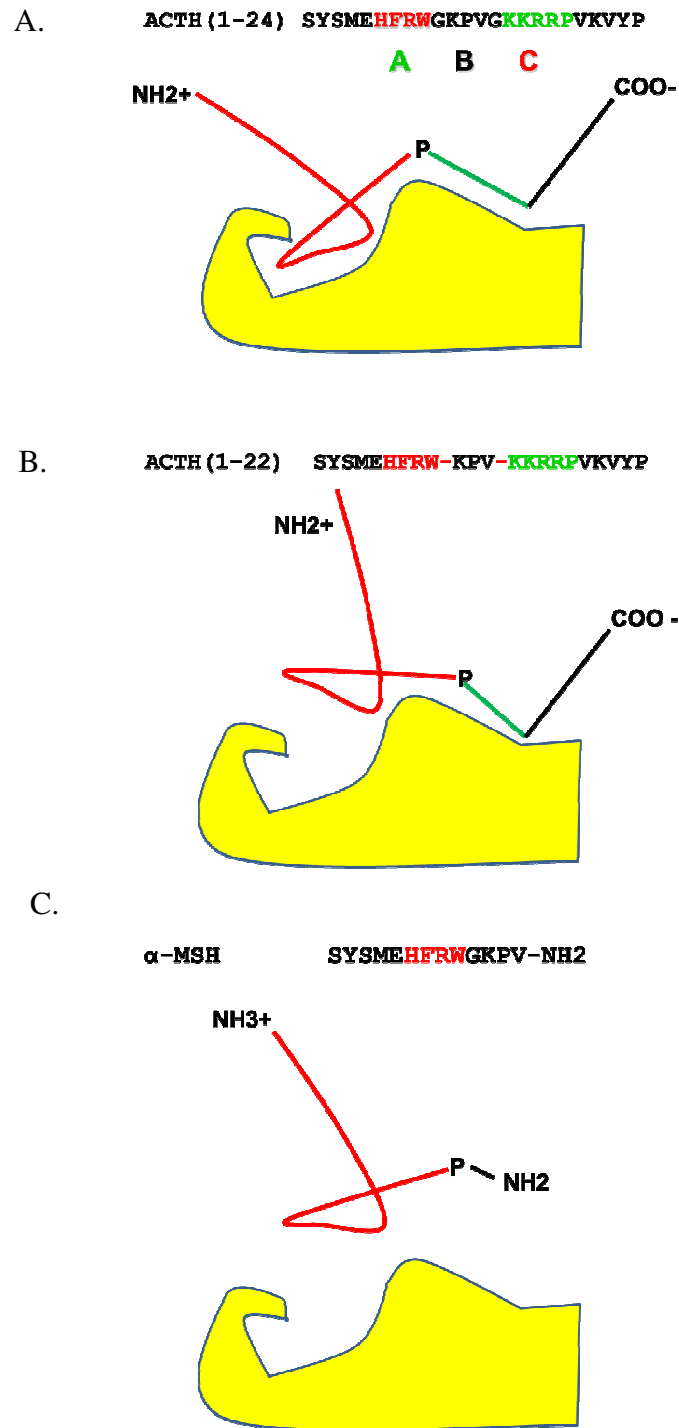


Figure 10. Schematic diagram for the model of hMC2R activation. A) ACTH(1-24) can bind to and activate hMC2R. B) ACTH(1-22) can bind to hMC2R but not activate it. C). α-MSH cannot bind to hMC2R.

## **CHAPTER TWO**

### **Identification of an ACTH Antagonist**

The primary role of ACTH is to stimulate the synthesis of glucocorticoids in adrenal cortex via melanocortin-2 receptor (Chan et al., 2011). Due to the presence of HFRW motif, which is the core sequence of melanocortin peptides (Schwyer, 1977), ACTH is capable of activating any other receptor in this gene family. As concluded in Chapter One, one of the distinct features of MC2R the need for both the HFRW and KKRRP motif in order to activate the receptor. By contrast,  $\alpha$ -MSH [Acetyl-ACTH(1-13)-amide] has the HFRW motif, but lacks the KKRRP motif, and as a result this ligand cannot activate MC2R. Collectively these data point to the region at position 14 to 24 of ACTH as a possible motif that could function as a competitive inhibitor of ACTH for MC2R. In this chapter, ligand competition and binding assays will be utilized to examine the potential of several truncated C-terminal analogs as ACTH antagonist that could selectively target hMC2R.

## **Results**

### **hMC2R cAMP Response Analysis**

To evaluate the direct response of activated receptor in transfected CHO cells, cAMP measurement was used in the experiments of this chapter. To begin with, we examined the cAMP response after 15-minute stimulation of ACTH(1-24), A5 analog, and KKRRP analog. In the presence of IBMX, these analogs were introduced to the cells individually, and then the cell lysate was harvested and assayed immediately using the direct cAMP enzyme immunoassay kit. As indicated in Figure 11A, the cAMP production in response to 100nM A5 analog was 200pmol, which was only 42% of the amount of cAMP produced by ACTH(1-24) stimuli ( $P < 0.5$ ). The cAMP level was undetectable when the cells were stimulated with 1nM A5 analog. Meanwhile, due to the lack of HFRW motif, analog KKRRP was unable to induce a cAMP response at concentration of 1nM or 100nM.

To examine whether HFRW and KKRRP motif that are present in separate peptide fragments are sufficient to activate hMC2R, the transfected CHO cells were incubated with 100nM KKRRP and 1 $\mu$ M  $\alpha$ -MSH or 100nM KKRRP and 1 $\mu$ M ACTH(4-10). There was no receptor activation detected based on the level of cAMP production (Figure 11B).

### **Ligand Competition cAMP Assay**

As discussed in Chapter One, the KKRRP motif is the secondary binding site of ACTH(1-24) for hMC2R. Consequently, ACTH(1-24) analogs containing KKRRP motif

but lacking HFRW motif should be able to function as a competitive inhibitor of this receptor. A series of C-terminus analogs (Sequences shown in Table 2) were synthesized and tested at a concentration of 1 $\mu$ M with the co-incubation of ACTH(1-24) ranging from 10<sup>-11</sup> to 10<sup>-7</sup>M. We first examined analog KPV, KKRRP and RRP (ACTH position 11-24, 15-24, and 17-24). The co-incubation of these analog with ACTH(1-24) led to the reduction of cAMP production at various degrees (Figure 12A). Analog KPV and RRP decreased the maximal cAMP response by 30% and 20% respectively, whereas analog KKRRP completely abolished the ACTH(1-24) induced cAMP response. Additionally, the KKRRP analog fully kept this inhibitory effect when the analog concentration applied to the cells was reduced from 1 $\mu$ M down to 100nM; at the concentration of 10nM, KKRRP could still block 35% of ACTH(1-24) induced cAMP response (Figure 12C). Thereafter, the inhibitory effect of two modified forms of KKRRP analogs was evaluated as shown in Figure 12B. Attachment of an acetyl group at the N-terminus of KKRRP analog (Ac-KKRRP) was not as an efficient antagonist as compared to KKRRP analog, but it retained 30% inhibition ability, while alanine substitution at position 20-24 (KKRRPAs) caused this analog to lose the ability to compete with ACTH(1-24) (Figure 12B).

### **Ligand Binding Affinity Analysis**

To determine the binding affinity of these truncated analogs, radioligand binding assay was performed using <sup>125</sup>I labeled ACTH(1-39) (Figure 12D). Compared with ACTH(1-24) ( $K_i = 1.2 \times 10^{-8} \text{M} \pm 8.4 \times 10^{-9} \text{M}$ ), a right shift in ACTH(1-39) binding curve

displayed with the co-incubation of analog RRP ( $K_i = 3.0 \times 10^{-7} \text{M} \pm 8.8 \times 10^{-8} \text{M}$ ) and KKRRPAs ( $K_i = 8.7 \times 10^{-8} \text{M} \pm 3.2 \times 10^{-8} \text{M}$ ), indicating their low binding affinity. In agreement with cAMP response analysis, analog KKRRP can bind to hMC2R most efficiently ( $K_i = 2.5 \times 10^{-8} \text{M} \pm 1.5 \times 10^{-8} \text{M}$ ) among these analogs.

## Discussion

The overproduction of ACTH(1-39) by the anterior pituitary gland, as a result of Cushing's Syndrome or due to chronic stress, leads to the over stimulation of MC2R on adrenal cortical cells and the subsequent overproduction of cortisol (Cushing, 1932; Arnaldi et al., 2003). In addition to the surgical resection of the underlying tumor, medications currently used in the therapies of Cushing's syndrome primarily target adrenocortical steroidogenesis (e.g., ketoconazole and metyrapone), ACTH secretion (e.g., bromocriptine and octreotide) or glucocorticoid receptor (e.g., mifepristone) (Tritos and Biller, 2012). Nevertheless, due to the lack of selectivity, administration of these medicines may induce many side-effects such as gastrointestinal toxicity, nausea, and hypertension (Tritos and Biller, 2012). In the development of effective medication for Cushing's Syndrome, one strategy to prevent systemic effects and enhance the selectivity to HPA axis would be to block the activation of MC2R by introducing an ACTH antagonist.

Previous studies reported that truncated analog ACTH(6-24) could bind to hMC2R that was expressed on OS3 adrenal cells, but completely lost the ability to

activate the receptor (Chen et al., 2007); ACTH(11-24) can act as a competitive inhibitor of ACTH(1-24) (Seelig et al., 1975). These observations shed a light to the identification of a ACTH antagonist that embedded within the sequence of ACTH(1-24). As discussed in Chapter One, HFRW motif is required in ACTH(1-24) to activate all melanocortin receptors; therefore, the absence of this domain is necessary in the sequence of ACTH antagonist. Moreover,  $\alpha$ -MSH (acetyl-ACTH(1-13) amide) is sufficient to activate MC1R, MC3R, MC4R and MC5R, but not to MC2R. In order to properly bind to MC2R, KKRRP motif (position 15-19) is required to be present in the ligand. Clearly, position 14-24 of ACTH(1-24) is a critical region to study that would lead to the selectivity of antagonist to MC2R. As a result, we evaluated the ability of competitive inhibition of several truncated ACTH analog in CHO cells expressing hMC2R and mMRAP1. Surprisingly, ACTH(15-24)/KKRRP was working as the best competitive inhibitor compared with ACTH(11-24)/KPV and ACTH(17-24)/RRP, at the concentration as low as  $10^{-8}$ M (Figure 12A, 12C). This finding was further supported by ligand binding affinity analysis (Figure 11A). Although alanine substitution at position 20-23 in ACTH(1-24) didn't affect the activation of hMC2R (Chapter One), same replacement performed in analog ACTH(15-24) did diminish the competition and binding ability of this analog (Figure 12B; 11), suggesting the last five position may play a role in the secondary structure of analog KKRRP that facilitates its binding to the receptor. Collectively, these observations suggested that ACTH(15-24) can act as an antagonist of ACTH(1-24) in our *in vitro* system.



Over the past decade, many pharmaceutical and biotech companies have drawn their attention to peptide drugs due to the distinctive advantages of this class of medications, such as high bioactivity and specificity, minimal drug-drug interactions, as well as low tissue accumulation and toxicity (Ayoub and Scheidegger, 2006). On the other hand, stability of peptides is their major disadvantages that limited the success of peptide drugs. The intrinsic properties of peptides could result in short half-life and low bioavailability issues when administered *in vivo* (Ayoub and Scheidegger, 2006). ACTH(15-24) is a short peptide with only ten amino acid residues. Hence, the main challenge it may encounter in serum or plasma would be the peptidase degradation predominantly by exopeptidases. To overcome this problem, the common approach is to protect the free N- and C-terminus with minor modifications. For example, one of the naturally modified hormones is  $\alpha$ -MSH; under the protection of both amino-acetylation and carboxy-amidation, it can only be degraded by endopeptidases *in vivo* (Marks et al., 1976). In the case of somatostatin analogs, N-terminal acetylation alone was able to expand the half-life from 3 minutes up to 400 minutes (Benuck and Marks, 1976). As a result, we evaluated the competition ability of ACTH(15-25) when attached with an acetyl group (analog Ac-KKRRP). Although this adjustment at N-terminus caused a partial lose in its antagonist function (Figure 12B), whether this analog could make a good drug candidate in *in vivo* system is waiting for further examinations.

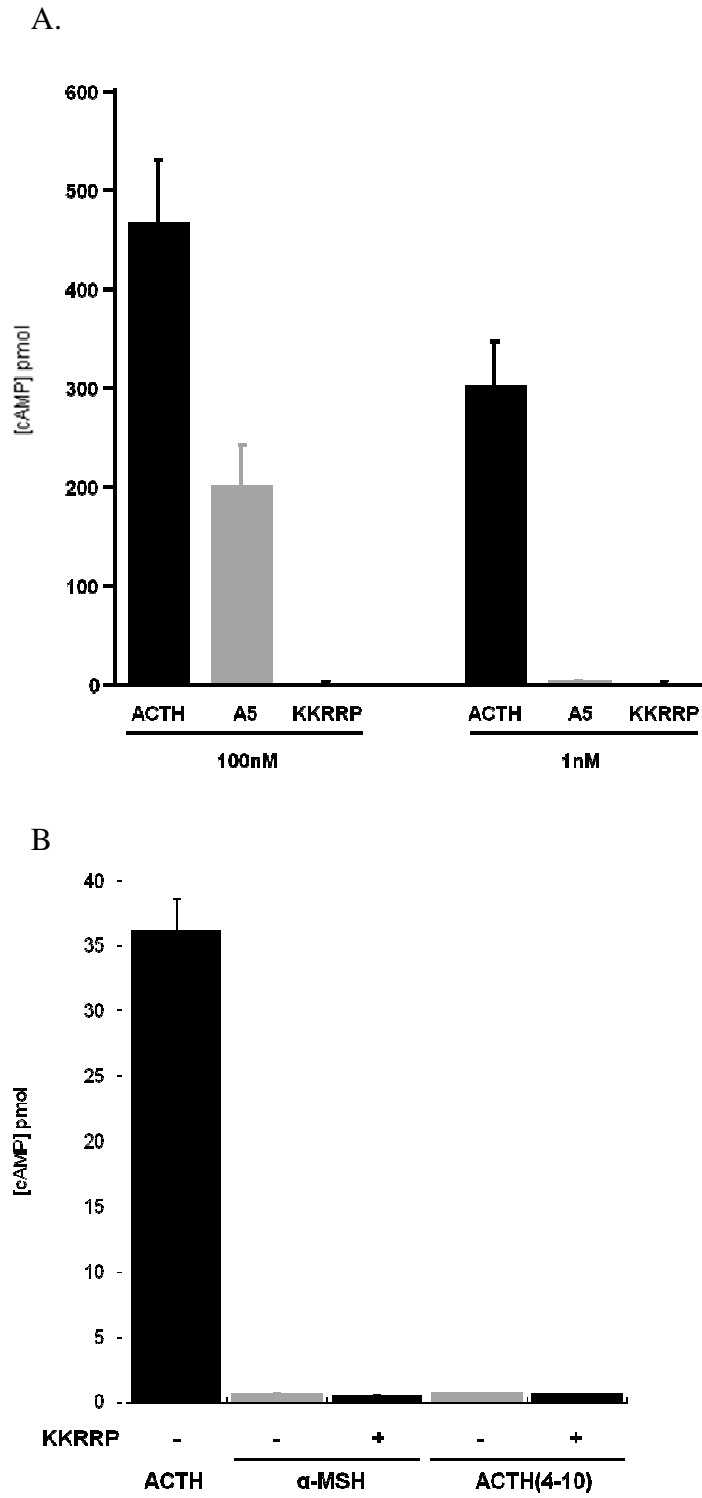


Figure 11. cAMP response in transfected CHO cells after 15-minute stimulation of A). ACTH(1-24), A5, or KKRRP; B). KKRRP/ $\alpha$ -MSH or KKRRP/ACTH(4-10)

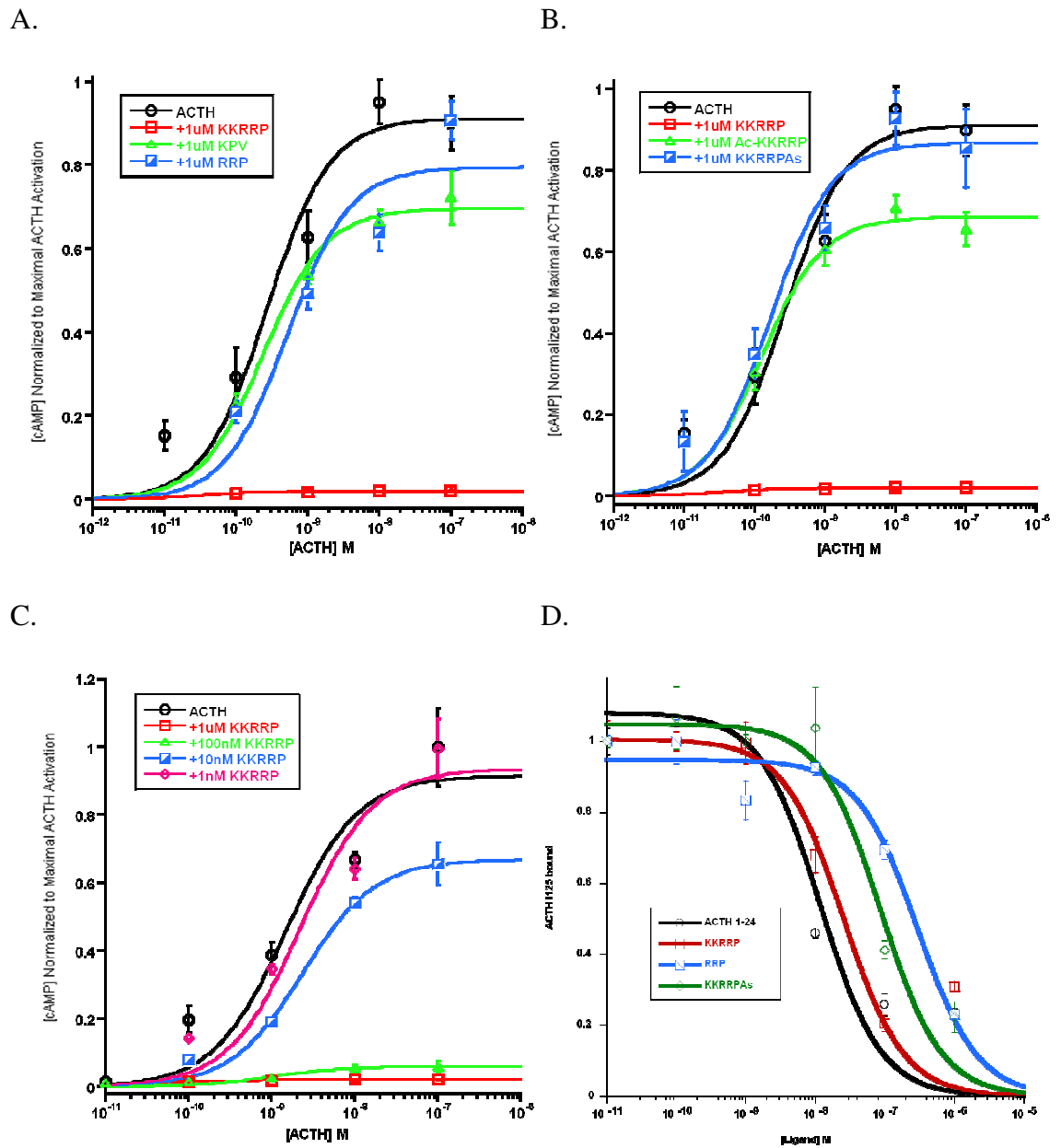


Figure 12. ACTH(1-24) dose response curves with co-incubation of C-terminal truncated analogs: A) 1 $\mu$ M of KPV, KKRRP and RRP; B) 1 $\mu$ M of KKRRP, Ac-KKRRP and KKRRPAs; C) 1nM to 1 $\mu$ M KKRRP. D) Binding affinity of ACTH analogs at hsMC2R.

## **CHAPTER THREE**

### **Site-directed Mutagenesis of TM4, EC2, TM5 in Human MC2R**

Extensive studies have been performed to examine the molecular basis responsible for ligand binding and signaling in MC1R, MC3R, and MC4R (Yang et al., 2000; Chen et al., 2006), and conserved residues in transmembrane domains of these receptors have emerged as key elements that facilitate both  $\alpha$ -MSH binding and receptor activation (Haskell-Luevano et al, 1996). As reported by Pogozeva and colleagues, ten residues in hMC4R that are involved in the binding of HFRW motif distributed in TM2, TM3, TM6 and TM7 (Table 5 and Figure 13). Note that 90% of these residues are also present at the same position in other melanocortin receptors of humans (MC1R, MC3R and MC5R), and at least 70% of these residues are conserved in human, frog and rainbow trout MC2R orthologs (Table 5). Since HFRW motif is required for the activation of all melanocortin receptors (Schwyzer, 1977), it is reasonable to propose that MC2Rs use a similar binding mechanism (TM2, TM3, TM6 and TM7) for the HFRW motif of ACTH(1-24).

As shown in the sequence alignment in Figure 13, the differences in primary sequence between human MC2R and MC4R are primarily in two aspects: 1). The amino acid sequences of N-terminus, C-terminus, and IC3 in MC4R are significantly longer as

compared with MC2R. 2). Primary sequence identity of TM1, EC1, TM4, EC2, and TM5 is less than 45% between MC4R and MC2R. These observations are consistent with Pogozheva's hypothesis that none of the proposed HFRW binding regions fall into these domains. The low sequence identity of these domains may be the key to understand the recognition mechanism which determines the ligand selectivity of MC2R—a secondary binding site other than the HFRW motif to bind to, that allows this receptor to exclude  $\alpha$ -MSH while accepting ACTH(1-24).

Assuming that the 3-dimensional structure of MC2R orients the seven transmembrane regions in a barrel conformation as shown in the schematic diagram in Figure 14, then TM2, TM3, TM6, and TM7 would be in close proximity to interact with HFRW motif, and EC2 could be positioned in the KKRRP binding site flanked by TM4 and TM5. After a careful comparison between the primary sequences of these domains in difference species, we found that between the highly conserved intracellular loop2 and the cytosolic region of TM5, there is a sharp dichotomy distinguishing MC2R primary sequence from all other MCRs (Sequences alignment shown in Figure 15). Due to the extremely low primary sequence identity of this continuous domain in MC2R orthologs, EC2 with the flanking regions in TM4 and TM5 is likely the responsible domain for creating a unique binding site for KKRRP motif in MC2Rs.

In this Chapter, 23 mutant hMC2R were synthesized for evaluating the importance of this domain from G162 to P183 (See diagrams in Figure 5), in terms of both receptor activation and plasma membrane expression levels. Since alanine is the generally accepted amino acid of choice for mutagenesis substitution in this type of

analysis (Yang et al., 2000), individual alanine substitution was used for replacing original amino acid residues in mutagenesis in order to avoid disrupting receptor tertiary structure. In the presence of mMRAP1, cAMP luciferase reporter assay was performed to analyze the activation activity of these mutant hMC2Rs. All mutant receptors were tested using hACTH(1-24) at concentrations ranging from  $10^{-13}$ M to  $10^{-6}$ M. A wild type hMC2R was used as the positive control for each mutant receptor experiment. The basal luciferase activity of each receptor was subtracted from the luminescence readings individually. Then the dose response curves were plotted on a logarithmic graph using the Michaelis-Menton equation (Kaleidograph software).  $EC_{50}$  values of ACTH(1-24)-mediated response curves were used as the parameter to compare receptor activation levels. Statistical difference of  $EC_{50}$  values between wild type hMC2R and mutants were determined using unpaired Student t-test with equal variances. Significant difference was confirmed when  $P < 0.05$ . After ACTH signaling defect mutants were identified, cell surface ELISA was performed on these receptors in the presence of mMRAP1 to evaluate their trafficking efficiency.

## **Results**

### **Effects of Single Alanine Substitution in hMC2R for ACTH Signaling**

In this study, alanine substitution was individually introduced to positions of interest in hMC2R, including six amino acid residues in TM4 (G162- I167), seven residues in TM5 (T177-P183), and the entire EC2 (F168-I176). We expressed the mutant

receptor in CHO cells in the presence of mMRAP1, and the functions of these mutants were evaluated using cAMP responsive reporter assay. As summarized in Table 6, in 22 single mutant hMC2Rs we examined, 16 mutant receptors showed no changes in the ACTH(1-24)-mediated response curves as compared with wild type hMC2R, suggesting that alanine substitution at these positions did not affect hMC2R/ACTH interaction and receptor activation. Meanwhile, we identified six mutant receptors that displayed significant decreases in ACTH(1-24)-mediated responses ( $P < 0.05$ ). These positions are T164, F168, S169, H170, F178 and L181 (Table 6).

Within six TM4 mutants we examined, only one position, T164, had a significant negative effect on ACTH dose response curve (Figure 16). The  $EC_{50}$  of ACTH(1-24) for mutant T164 was  $(7.5 \pm 1.59) \times 10^{-12}$  M as compared to a  $EC_{50}$  of  $(2.7 \pm 1.23) \times 10^{-12}$  M for wild type hMC2R ( $P = 0.038$ ) (Table 6). Of 9 residues in the domain of EC2, alanine substitution on three successive positions that are located at the very beginning of this loop displayed significant reduction in ACTH(1-24) responses (Figure 17A). The  $EC_{50}$  values for these mutants, F168, S169 and H170, were  $(1.4 \pm 0.4) \times 10^{-11}$  M,  $(1.7 \pm 0.5) \times 10^{-11}$  M, and  $(6.1 \pm 2.0) \times 10^{-11}$  M, which were approximately 4-fold, 5-fold, and 16-fold of the  $EC_{50}$  of the wild type hMC2R ( $P = 0.03$ ,  $0.02$ , and  $0.01$ , respectively) (Table 6). Clearly, H170 was the most important site in EC2 that mediated receptor activation. In addition, positions F178 and L181 identified from TM5 mutants had even greater effects than all positions tested in TM4 and majority in EC2 (Figure 18). As indicated in Table 6, the  $EC_{50}$  values for F178 and L181 were  $(6.6 \pm 1.0) \times 10^{-10}$  M and  $(3.7 \pm 1.4) \times 10^{-11}$  M, which were approximately 174-fold and 10-fold of wild type hMC2R  $EC_{50}$ . Hence, mutant F178

in TM5 produced the greatest right-shift in ACTH(1-24) dose response curve (Figure 18A), and this position is likely one of the core residues that mediate the receptor/ligand interaction of hMC2R.

Next, we put alanine substitutions on both significant phenylalanine positions F168 and F178, and examined the activity function of this mutant (Figure 19A). Albeit  $EC_{50}$  of mutant FF was  $(9.2 \pm 2.1) \times 10^{-8}$  M and ACTH dose response curve was greatly shift to the right, this receptor kept full activation ability when stimulated with  $10^{-6}$  M ACTH, indicating that double substitution of F168 and F178 cannot completely block ACTH signaling. Hence, there are other positions involved in this binding event. Finally, we substituted all six significant positions (T164, F168, S169, H170, F168 and F178) with alanine residues to make an hMC2R 6A mutant, and this mutant completely lost the activation activity (Figure 19B)

### **Effects of Single Alanine Substitution in hMC2R for Receptor Trafficking**

In order to eliminate the possibility of trafficking defects introduced by alanine mutations, we co-transfected CHO cells with mMRAP1 and individual activation defect mutant hMC2R identified by cAMP luciferase reporter assay, and performed cell surface ELISA to evaluate the plasma membrane expression levels of these mutants. All experiments were executed in triplicates. Data were first subtracted by the background signals, converted to the ratio of plasma membrane expression to total expression, and then normalized to wild type hMC2R. As indicated in Figure 20, albeit mutant S169 and L181 showed a lower level of surface expression as compared with wild type hMC2R,



Student t-test analysis showed that none of these activation defect mutants demonstrated significant trafficking impairment.

## **Discussion**

By virtue of the functionally important residues that were previously identified in MC1, MC3, and MC4R, nine residues in MC2R were found to be responsible for ACTH binding and signaling (Chen et al., 2007). Within these positions, seven residues are located in TM2, TM3, TM6 and TM7, which is the proposed HFRW motif binding site identified from MC4R (Pogozheva et al., 2005). Not surprisingly, the other two residues in these positions, F168 in EC2 and F178 in TM5, are both unique amino acids in MC2R, and their position fall out of the proposed binding pocket for HFRW motif (Chen et al., 2007). As discussed in the ligand binding studies in Chapter One, the recognition sites for HFRW and KKRRP motif on ACTH(1-24) must span two portions in the receptor. Based on the 3-D structure of receptor (Figure 14), if the HFRW motif is positioned between TM2, TM3, TM6 and TM7, then the logical location for the KKRRP motif to bind must be present in TM4, EC2, and TM5. These observations supported our hypothesis that TM4, EC2 and TM5 may indirectly interact with the KKRRP motif, which in turn determine the ligand selectivity of MC2R.

In this Chapter, we systematically characterized the functional expression of 22 single-mutation hMC2Rs from position G162 to P183, which cover the entire EC2 and the flanking regions in TM4 and TM5. As indicated in Table 6 and Figure 16-18, 6

mutants, T164, F168, S169, H170, F178, and L181, caused significant decrease in ACTH response curve while trafficking defects were undetectable in plasma membrane expression analysis (Figure 20). In a previous study based on type 1 FDG mutant MC2R cases, Chung and colleagues identified six trafficking-competent mutations with ACTH signaling defects (D20N, 144M, D103N, D107N, R128C, and H170L) from 24 naturally occurring missense mutations found in these patients (Figure 21A; Chung et al., 2008). D107N and R128C are located in TM3, providing the evidence for the importance of this transmembrane domain in the interaction between MC2R and ACTH. These findings also supported Pogosheva's hypothesis that the binding site for HFRW motif in MCRs is between TM2, TM3, TM6 and TM7. Note that only one position (H170L) was detected in the region we are focusing on (TM4/EC2/TM5). Other than residue H170, two phenylalanine residues F168 and F178 were the previously reported by Chen's group (Chen et al., 2007). Our receptor activation results from double phenylalanine mutation (FF) indicated that removing these residues at the same time cannot completely block ACTH signaling (Figure 19A). Hence, there must be more residues involved in this process, and it appears to be position T164, S169 and L181 as we identified from our target region. In addition, these residues were the first time to be discovered that impaired ACTH(1-24) signaling without disrupting human MC2R trafficking in the presence of mMRAP1.

Taken together, the distribution of these essential residues is as follows: one position in TM4, two discrete positions in TM5, and three successive positions located at the beginning of EC2 (Summarized in Figure 21B). Notably, hMC2R has a relatively

short extracellular loop 2, which is composed of only nine amino acids. Thus, 33% residues of EC2 are involved in the binding of ACTH, suggesting a critical role of this loop playing in the proposed KKRRP motif binding region. When these positions were substituted by alanine residues at the time, the ACTH(1-24) was completely blocked. In order to confirm this effect was solely induced by the inactivated binding sites but not due to the trafficking defects of this mutant, the receptor cell surface expression analysis needs to be done in the future.

As described in Chapter One, our model for the binding of ACTH to MC2R is that the ligand first makes contact with the receptor at a KKRRP binding site and then activation is possible when the HFRW motif of the ligand is inserted into the corresponding binding site on the receptor. If the primary binding site for KKRRP motif is disabled in MC2R, then ACTH(1-24) would be rejected by this receptor just as  $\alpha$ -MSH. Would this alternation completely prevent the binding of ACTH(1-24) to the receptor? Would it eliminate the ACTH response of MC2R, or just take away its ligand selectivity? In this regard, a mutant hMC2R with alanine substitutions at all six residues identified in this Chapter will be examined in future studies.

Table 5. Proposed HFRW binding sites in MC4R and corresponding residues of MC2R.

AA	Region	MC4R			MC2R		
		H	X	RT	H	X	RT
E100	TM2	+	+	+	+	+	+
D122	TM3	+	+	+	+	+	+
D126	TM3	+	+	+	+	+	+
I129	TM3	+	+	+	-	-	-
L133	TM3	+	+	+	+	+	-
W258	TM6	+	+	+	+	+	+
F261	TM6	+	+	+	+	+	+
H264	TM6	+	+	+	+	+	+
N285	TM7	+	+	+	-	+	-
L288	TM7	+	+	+	-	-	+

H: Human; X: *Xenopus tropicalis*; RT: Rainbow trout (*Onchorynchus mykiss*)

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                                N-terminal                                [-----TM1-----]
hMC2R  MK-----HIINSYENINNTARNNSDCPRVVLPEEIFFTISIVGVLENLIVLLAVF
hMC4R  MVNSTHRGMHTSLHLWNRSSYRLHSNASESLGKGYSDDGGCYEQLFVSPEVFVTLGVISLLENILVIVAIA

                                IC1    [-----TM2-----]    EC1    [-----TM3-----]
hMC2R  KNKNLQAPMYFFICSLAISDMLGSLYKILENILLIRNMGYLKPRGSFETTADDIIDSFLVLSLLGSIFS
hMC4R  KNKNLHSPMYFFICSLAVADMLVSVSNGSETIVITLLNSTD-TDAQSFTVNIDNVIDSVICSSLLASICS
                                *                                *    *    *    *

--]    IC2    [-----TM4-----]    EC2    [-----TM5-----]
hMC2R  LSVIAADRYITIFHALRYHSIVTMRRTVVVLTVIWTFTGTGITMVIFSHHVPTVITFTSLFPLMLVFIL
hMC4R  LLSIAVDRYFTIFYALQYHNIMTVKRVGIIISCIWAACTVSGILFIYSDSSAVIICLITMFFTMLALMA

-----]    IC3    [-----TM6-----]    EC3
hMC2R  CLYVHMFLARSHTRKISTLP-----RANMKGAITLTILLGVFIFCWAPFVLHVLLMTFCPSNPYCAC
hMC4R  SLYVHMFLMARLHIKRIAVLEPGTGAIROGANMKGAITLTILLGVFVVCWAPFFLHLIFYISCPQNPYCVC
                                *    *    *

[-----TM7-----]    C-terminal
hMC2R  YMSLFQVNGMLIMCNAVIDPFIYAFRSPELRDAFKKMIFCSRYW
hMC4R  FMSHFNLYLILIMCNSIIDPLIYALRSQELRKTFKEI-ICCYPLGGLCDLSSRY
                                *    *

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Figure 13. Sequence alignment of human MC2R and MC4R. Positions shaded indicate identical amino acids in these receptors. \* indicates proposed amino acids that are involved in HFRW binding sites in MC4R as reported by Pogosheva.

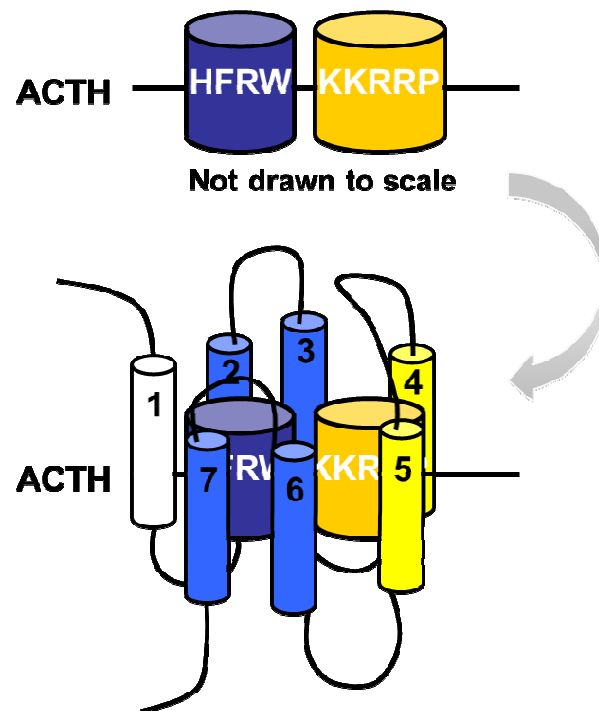


Figure 14. Schematic diagram of MC2R proposed binding sites for HFRW motif and KKRRP motif.

	[----Mutant region---]																								
	IC2										TM4					EC2					TM5				
hMC2R	IAADRYITIFHALRYHSIVTMRRTVVVLTVITFTCTGTGITMVIFSHHVPTVITFTSLFPLMLVFLICLYVHMF																								
xMC2R	IAADRYITVFHALHYHNIMTVKR SVILAVITVFCGSGSIAIIMLFHDTAMICI LTVMFLLLLVLIVCLYIHMF																								
rMC2R	IA DRYVTIFHALRYHNIMTTRR AAALAGI WALCGVAGAVMVAFCDATVIKIFF VLF LISL LLLIFLYVHMF																								
zMC2R	IA DRYISIFHALRYHMLTMRRLIILFTI WVLCGTS GALMVGFEEAATVTIFF VLF TALL L LLLYVHMF																								
hMC1R	IA DRYISIFYALRYHSIVTLPR RRAVA AIWASVVFSTLFIAYYDHVAVLLCLVVFFLAMLVL AVLYVHML																								
xMC1R	IAIDRYITIFYALRYHSIMTLRRVVIA GVIVSVSLVCAAIFIVYHE RAVILCL VFFLFMLAL VALYIHMF																								
zMC1R	IAADRYITIFYALRYHSIMTQR VGI LVVWLASITSSSLFIVYHTDNVAIACLVTFFGVTLVFTAVLYLHMF																								
hMC3R	IA DRYVTIFYALRYHSIMTVRK LTL VAIWCCGVCGVFIVYSE K MVIVCL TMF AMMLL GTLYVHMF																								
xMC3R	IAIDRYITIFYALRYHSIMTVKK IAL VVIWTS CII CGIVFIVFSE KTVIVCL TMF TMLVL ATMYVHMF																								
zMC3R	IA DRYVTIFYALRYHSIVTVRR LVA AVIWLVCVCGIVFIVYSE KTVIVCL TMF AMLVL ATLYVHMF																								
hMC4R	IA DRYFTIFYALQYHNIMTVKRVGII SCIIWA ACTVSGILFIIYS D SAVIICL TMF TMLAL ASLYVHMF																								
xMC4R	IA DRYFTIFYALQYHNITVRR VVI SCIIW TACSISGVLFI IYD AVVIICL SII TMLAL ASLYVHMF																								
zMC4R	IA DRYITIFYALRYHNIMTQRR GTI TCIWTFCTVSGVLFIYSE TTVLICL SMF TMLAL ASLYVHMF																								
hMC5R	IA DRYVTIFYALRYHHIMTARRSGAI AGIWA FCTGCGIVF ILYSE TYVILCL SMF AMFLFLVSLYIHMF																								
xMC5R	IA DRYVTIFYALRYHNIMTMR R GII ACIWT FCTGCGIIF ILYYE TYVIICL TMF TMLFL VSLYIHMF																								
zMC5R	IA DRYVTIFYALRYHNIMTVRR ALI GGIWTFCTGCGIVFI IYS DNTSVIVCLVSMF IMLAL ASLYSHMF																								

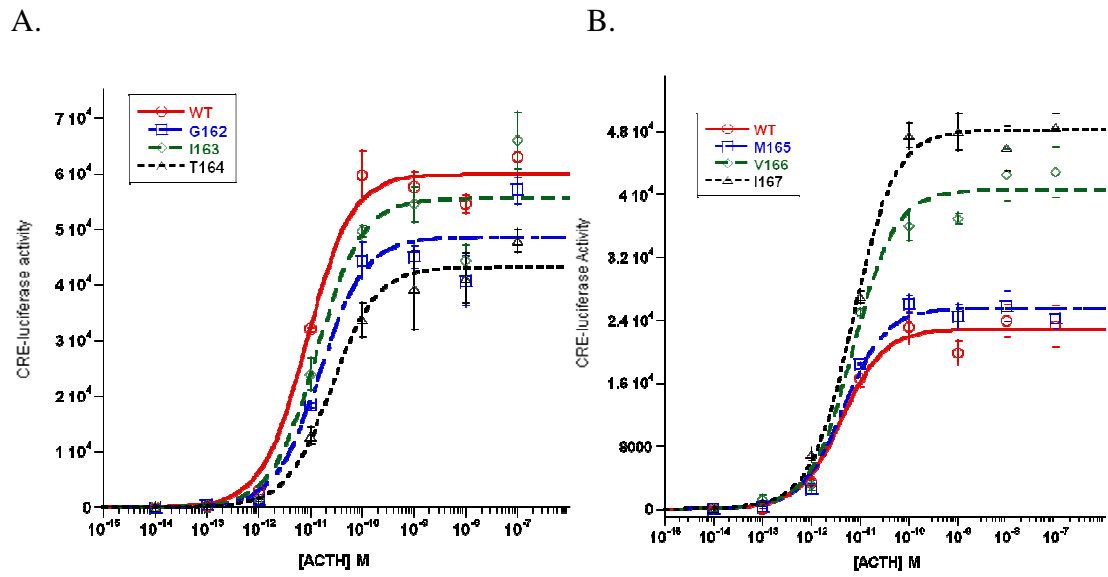


Figure 16. ACTH(1-24) dose response curves of TM4 mutant hMC2Rs in the presence of mMRAP1.



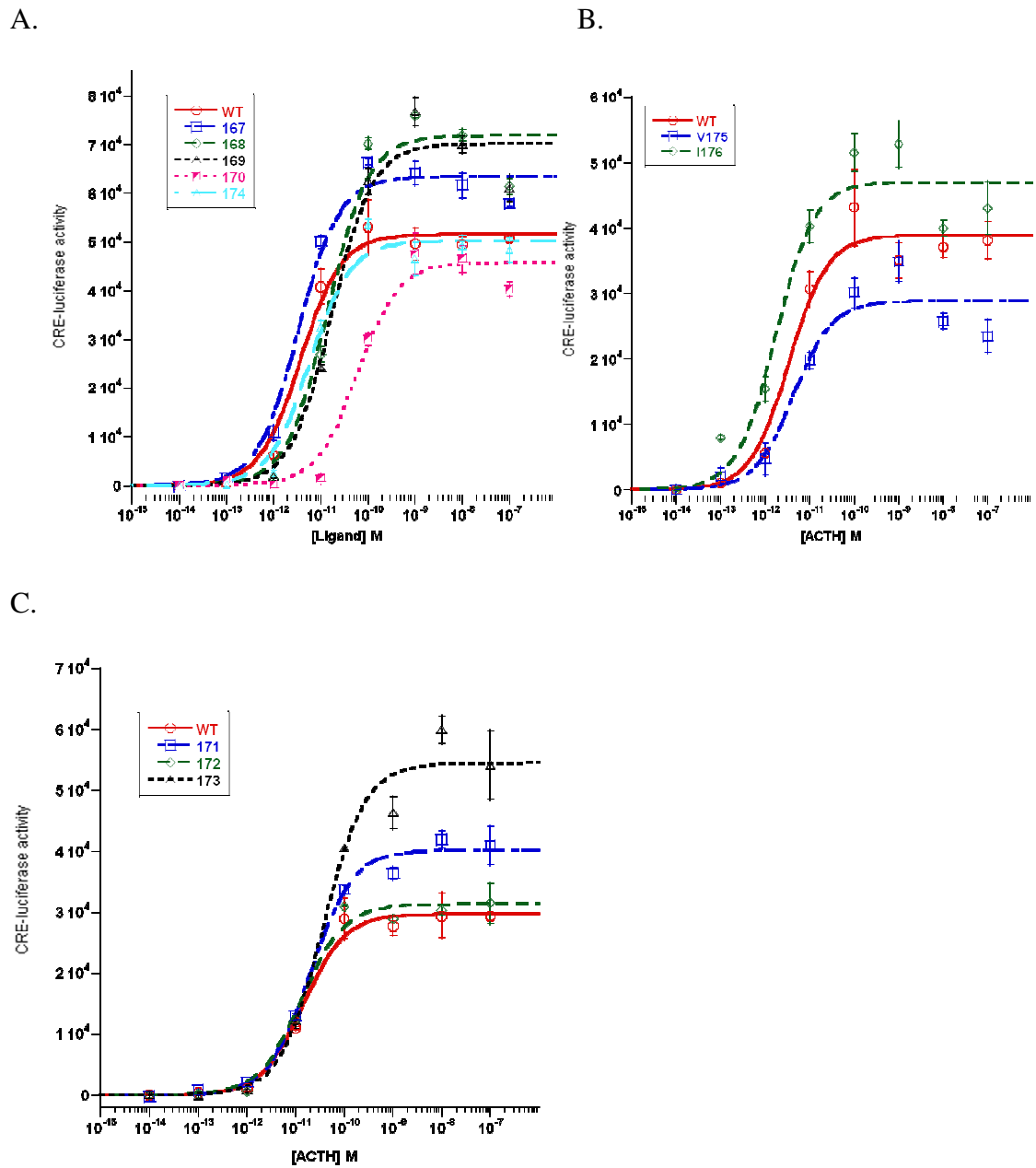


Figure 17. ACTH(1-24) dose response curves of EC2 mutant hMC2Rs in the presence of mMRAP1.

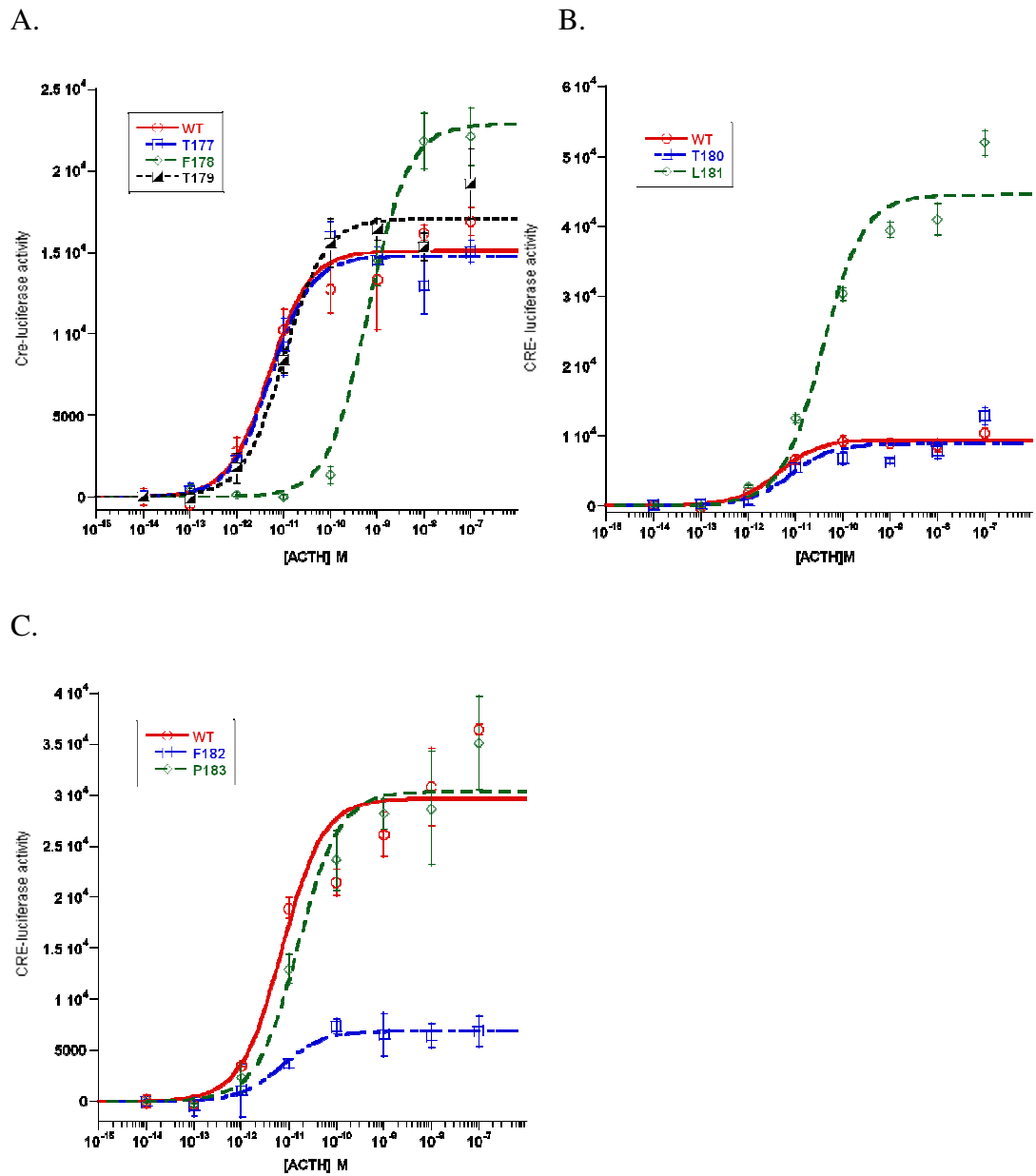
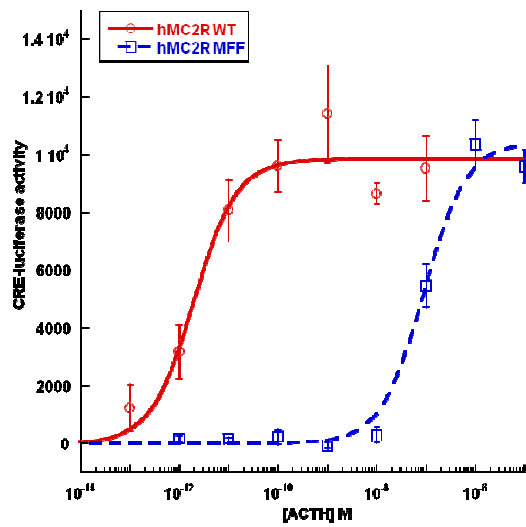


Figure 18. A-C) ACTH(1-24) dose response curves of TM5 mutant hMC2Rs in the presence of mMRAP1.

A.



B.

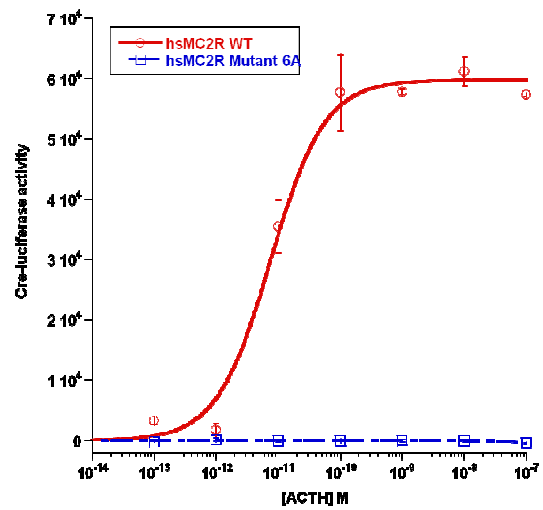


Figure 19. A) ACTH(1-24) dose response curves of hMC2R FF mutant (F168 & F178). B) ACTH(1-24) dose response curves of hMC2R 6A mutant

Table 6. EC<sub>50</sub> values of mutant hMC2R.

Domain	Alanine Substitution	EC <sub>50</sub> ± SEM (10 <sup>-12</sup> M)	P Values
WT	N/A	3.8 ± 0.3	N/A
TM4	G162	4.5 ± 1.79	0.23
	I163	4.3 ± 1.15	0.20
	T164	7.5 ± 1.59*	0.038
	M165	4.6 ± 0.98	0.42
	V166	7.2 ± 1.6	0.10
	I167	3.4 ± 0.8	0.60
EC2	F168	14.1 ± 4.6*	0.03
	S169	17.2 ± 5.1*	0.02
	H170	60.9 ± 20.3*	0.01
	H171	5.0 ± 0.61	0.08
	V172	3.3 ± 3.87	0.40
	P173	4.8 ± 1.02	0.13
	T174	5.9 ± 1.7	0.10
	V175	4.4 ± 2.4	0.4
TM5	I176	1.7 ± 0.8	0.2
	T177	5.4 ± 1.60	0.44
	F178	660 ± 100**	0.0014
	T179	10 ± 2.7	0.099
	S180	8.1 ± 7.4	0.33
	L181	37 ± 14*	0.041
	F182	4.2 ± 1.5	0.51
	P183	9.1 ± 3.3	0.13
Double Mutation	F168 & F178 (FF)	92000 ± 21000*	0.006
Sextuple Mutation	T164, F168, S169, H170, F168, F178 (6A)	N/A	

EC<sub>50</sub> values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (\*:P<0.05, \*\*:P<0.005). N/A: No activation.

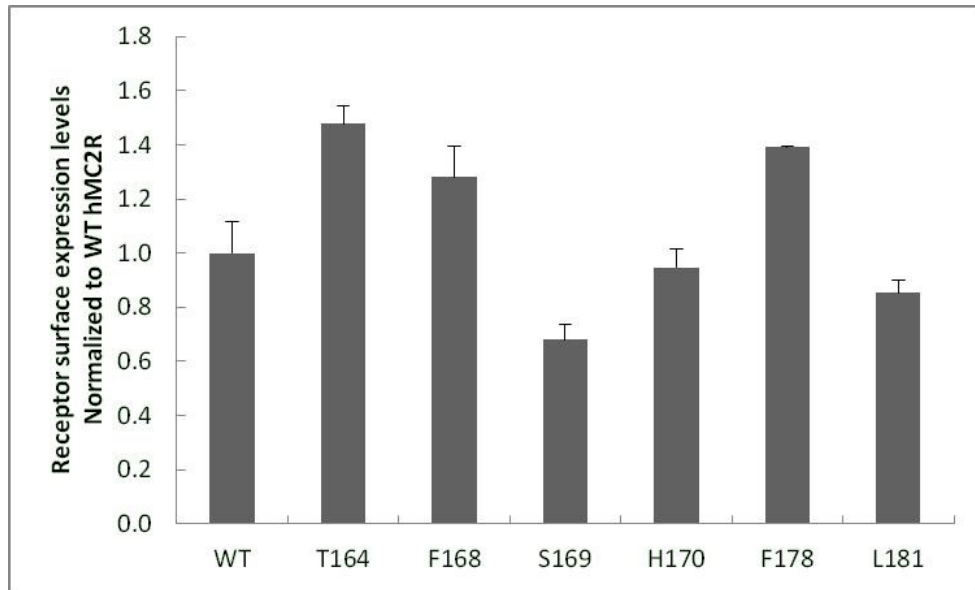
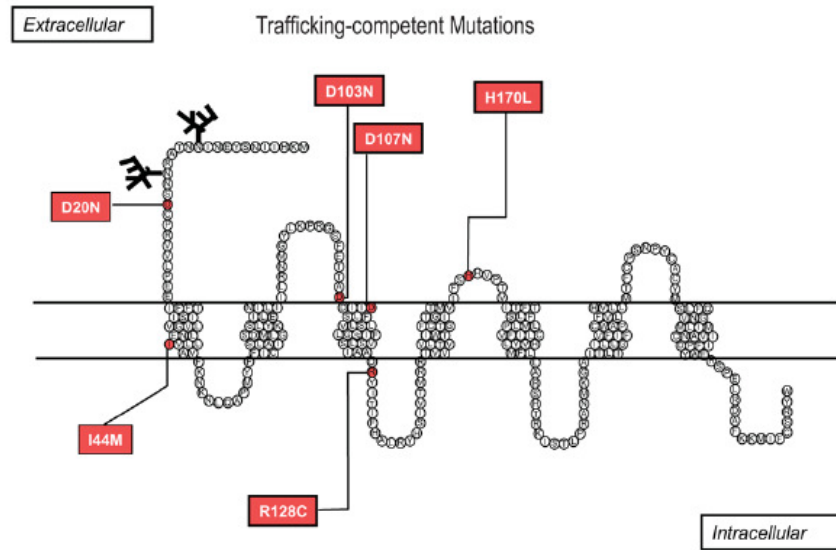


Figure 20. Cell surface expression analysis of ACTH signaling defect mutants.

A.



B.

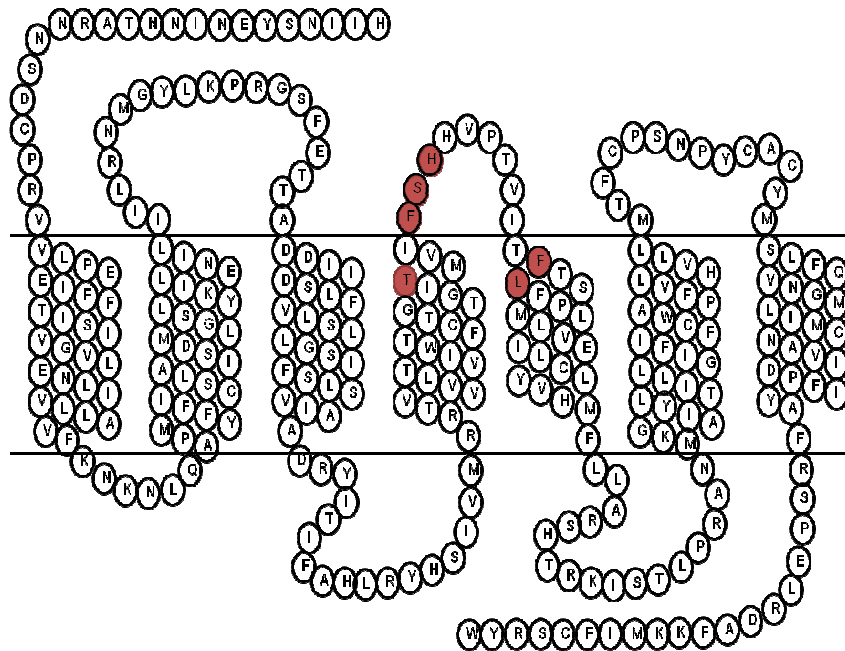


Figure 21. Schematic plot of the MC2R mapping the locations of A) 6 trafficking-competent mutations identified from Type 1 FDG (Chung et al., 2008). B) 6 residues identified in this study for the proposed KKRRP motif binding site.

## CHAPTER FOUR

### Ligand Selectivity Properties of Rainbow Trout Melanocortin-2 Receptor

According to the 2R hypothesis, the first gene duplication of ancestral MCR gene yielded two paralogous genes (MC<sub>a</sub> and MC<sub>b</sub> receptors) in agnathan vertebrates, and then the second duplication event produced four paralogous genes (MC1R, MC5/2R, MC3R and MC4R) in ancestral gnathostomes (Vastermark and Schioth, 2011). As to the fifth paralogous gene MC2R, which could be activated only by ACTH, appeared in the ancestral bony fishes and has since radiated throughout the extant bony vertebrate classes (Schioth et al., 2005). Recent studies revealed that a MC2R ortholog cloned from cartilaginous fish Elephant shark could be transported to plasma membrane in the absence of MRAP1, and it is the only MC2R currently identified that can be activated by ACTH and MSHs (Reinick et al., 2012). These observations indicated that MC2R dependence on MRAP1 for functional activation and the exclusive selectivity of this melanocortin receptor for ACTH are features that emerged after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes more than 400 million years ago. Therefore, understanding the ligand selectivity features of a bony fish MC2R would be an essential component in modeling the evolution MC2R activation. In this study, rainbow trout (*Oncorhynchus mykiss*) MC2R (rtMC2R) was selected for demonstrating

teleost MC2R activation. Like the mammalian MC2Rs, rtMC2R requires the presence of MRAP1 to function properly (Liang et al., 2011). In the co-evolution of MC2R and MRAP1 during the radiation of vertebrates, it appears that at least a tetrapod branch and a bony fish branch have emerged with respect to the interaction between MC2R and MRAP1 (Liang et al., 2011). Since we started this project when the genes encoding rainbow trout MRAPs had not been identified yet, zebra fish MRAP1 (zfMRAP1) was co-expressed with rtMC2R within CHO cells in order to obtain the optimal ACTH responses.

In the analysis of hACTH(1-24) and hMC2R interactions (Chapter One), amino acid residues within Zone A, Zone B, and Zone C were involved in ACTH binding and signaling to varying degrees. To evaluate the functions of these domains in the activation activities of rtMC2R, we did a comparative study using the same analogs as Chapter One (See sequences in Table 2): (1) Zone A analogs—H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup>; (2) Zone B analogs—G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup>; and (3) Zone C analogs—R<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup>P<sup>19</sup>. Note that the position 15 of rtACTH(1-24) as well as many other vertebrate species is an arginine (R<sup>15</sup>), instead of a lysine (K<sup>15</sup>) in hACTH(1-24) (Figure 22), hence it is referred as R/KKRRP motif for this Chapter. Both of these basic amino acids are generally considered to be interchangeable and able to readily substitute for one another. In addition, it has been reported that hACTH(1-24) can fully stimulate rtMC2R that are expressed in CHO cells in the presence of zfMRAP1 (Liang et al., 2011). Hence, hACTH(1-24) was used in this study as the wild-type rtACTH(1-24).



## Results

### Ligand Selectivity of rtMC2R

#### *Zone A analogs*

Five Zone A analogs (See sequences in table 2) were applied individually to CHO cells transiently transfected with rtMC2R and zfMRAP1, and CRE-Luc activity was measured after 4 hours of stimulation (Figure 23A). Consistent with the parallel experiment on hMC2R, replacing the entire HFRW motif abolished rtMC2R response completely. Nevertheless, the importance of the four positions in Zone A with respect to the activation of rtMC2R was different as compared to hMC2R. Firstly, all four analogs (AFRW, HARW, HRAW, and HFRA) greatly diminished rtMC2R activation with a 23-fold, 49-fold, 1400-fold, and 681-fold increase in  $EC_{50}$  values (Table 7), whereas for hMC2R, ARFW analog did not significantly alter the dose response curve. Secondly, analog HFRA was able to stimulate weak rtMC2R activation at the concentrations higher than  $10^{-7}M$ , whereas the same analog failed to activate hMC2R. Finally, the order of effectiveness of these positions affecting rtMC2R activation is  $R^8 > W^9 > F^7 > H^6$ .

#### *Zone C analogs*

To determine the role of R/KKRRP motif on the activation of rtMC2R, CHO cells expressing rtMC2R and zfMRAP1 were challenged with three Zone C analogs respectively (See sequences in Table 2). In the absence of R/KKRRP motif, analog A5 failed to promote any stimulatory effect on rtMC2R even at concentration of  $10^{-6}M$

(Figure 23B), clearly indicating that this motif is equally critical as HFRW motif.

Additionally, position R<sup>15</sup>K<sup>16</sup> and R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> were tested separately using analog AARRP and KKAAA. The EC<sub>50</sub> values of KKAAA and AARRP were significantly increased up to 1112-fold and 615-fold as compared with hACTH(1-24) (Table 7).

### ***Zone B analogs***

The importance of the region between HFRW and KKRRP was first examined using four ACTH(1-24) analogs with alanine substitutions at G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup>, G<sup>10</sup>G<sup>14</sup>, K<sup>11</sup>P<sup>12</sup>, and P<sup>12</sup> (Analog A10-14, AKPVA, GAAVG, and GKAVG in Table 2). Generally, alanine substitution at Zone B had greater effects on rtMC2R activation curves than hMC2R. As indicated in Figure 23C and Table 7, both A10-14 and AKPVK significantly reduced activation activity by 376-fold and 67-fold increase on EC<sub>50</sub> values, while analog GAAVG, and GKAVG also introduced a right shift in activation curve, albeit the EC<sub>50</sub> values were not statistically larger than hACTH(1-24). Two truncated Zone C analogs, ACTH(1-21) and ACTH(1-22), were also tested on rtMC2R. Note that skipping position G<sup>10</sup>G<sup>14</sup> or K<sup>11</sup>P<sup>12</sup>V<sup>13</sup> in Zone B induced a complete loss in hMC2R activation (Figure 6C). Similar scenario was observed in rtMC2R activation curves. Receptor activation in response to both ACTH(1-21) and ACTH(1-22) were undetectable when analog concentration was lower than 10<sup>-7</sup>M (Figure 23D).

## Discussion

As the core sequence of hACTH(1-24), HFRW motif plays an essential role in activating hMC2R, with the tryptophan at the last position that predominantly contributes to the function of this motif (Figure 6A). In the case of rtMC2R, A4 analog did not have any stimulatory effect even at concentration of  $10^{-6}$ M (Figure 23A), hence, HFRW motif is also required for the activation of rtMC2R. Nevertheless, the importance of each position within this region results in a slightly different scenario. Replacement of a single alanine at any of these positions significantly diminished the receptor activation activity, whereas none of them was able to completely abolish the stimulatory effect at higher doses (Figure 23A). It appears that histadine<sup>6</sup> and phenylalanine<sup>7</sup> that establish the reverse  $\beta$ -turn structure in  $\alpha$  MSH (Ying et al., 2003) are actually less important than tryptophan<sup>9</sup> and arginine<sup>8</sup> in the interaction of hACTH(1-24) and rtMC2R (Table 7). These observations are mostly consistent with hMC2R indicating that the side chains of tryptophan<sup>9</sup> and arginine<sup>8</sup> may play an essential role interacting with rtMC2R.

Next, we examined the effect of alanine substitutions at R/KKRRP motif on rtMC2R activation. As discussed in Chapter One, hMC2R recognizes the R/KKRRP motif in hACTH(1-24) as a significant binding site that determines its ligand selectivity property. Not surprisingly, alanine substitutions at entire KKRRP motif caused analog A5 fully lost the ability to stimulate rtMC2R (Figure 23B). Note that replacing the entire R/KKRRP motif with alanine is not sufficient to eliminate the stimulatory effect in human MC2R at concentration of  $10^{-6}$ M, whereas in rtMC2R ligand activation study, the

absence of receptor activity acquired from A5 analog dose response curve has been clearly supporting the secondary binding site hypothesis. Additionally, it appears that R<sup>15</sup>K<sup>16</sup> motif and R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> motif are equally contributing to this binding site (Figure 23A and Table 7).

Having confirmed the same activation sites HFRW motif and R/KKRRP motif for rtMC2R, we evaluated whether the G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup> sequence between these two motifs is as critical as it is in hMC2R. As compared to hMC2R, alanine substitutions at entire Zone B had less negative effects on rtMC2R activation curve, whereas individual substitution caused a greater right-shift in each curve (Figure 23C and Table 7). With regard to Zone B truncated analogs, albeit a weak activation activity appeared at concentration higher than 10<sup>-7</sup>M, both ACTH(1-22) and ACTH(1-21) greatly diminished rtMC2R responses (Figure 23D). Collectively, the relative distance between HFRW motif and R/KKRRP motif plays a more important role than the sequence of this region.

According to the proposed model for the interaction between ACTH(1-24) and MC2R (Figure 10), R/KKRRP motif first binds to MC2R, and HFRW motif is then positioned in its corresponding binding site, leading to the activation of receptor. In agreement of this hypothesis, our results obtained from rtMC2R indicated that R/KKRRP motif, HFRW motif, as well as the amino acid sequence in between are absolutely required for the activation of the activation of rtMC2R (Figure 23). As compared to the influence of these analogs on hMC2R activation, individual residues within these motifs may play a slightly different role in terms of the interaction with certain residues of the receptor, whereas the importance of three motifs was consistent in these receptors. A

comparative analysis between human and rainbow trout MC2R will be discussed in next chapter.

```

[-----ACTH (1-39) -----]
[-----ACTH (1-24) -----]
      [A ][ B ][ C ]
Human  SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEP
Chicken SYSMEHFRWGKPVGRKRRPIKVYPNGVDEESAESYPMEF
Gecko   SYAMEHFRWGKPVGRKRRPVRVYPNGVEEESSESYPQEF
Xenopus AYSMEHFRWGKPVGRKRRPIKVYPNGVEEESAENYPMEL
Trout   SYSMEHFRWGKPVGRKRRPVKVYTNGVEEESSEAFPSEM
Dogfish SYSMEHFRWGKPMGRKRRPIKVYPNSFEDESVENMGPEL

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Figure 22. Amino acid sequence alignment of ACTH in different species. Residues shaded in grey are sites at least 83% identical in all sequence.

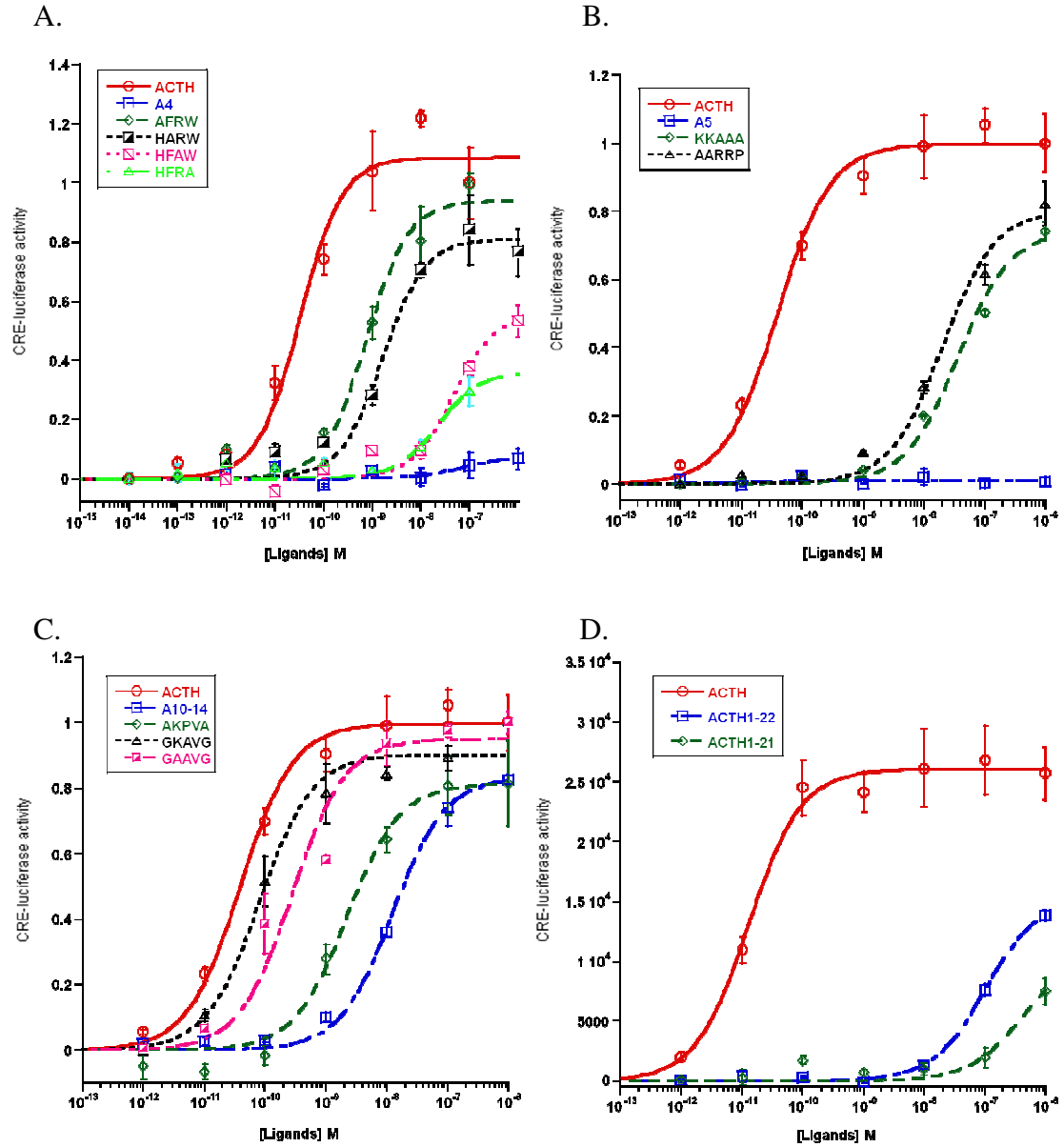


Figure 23. Activation curves of rtMC2R by the stimulation of hACTH(1-24) analogs: A) Zone A analogs; B) Zone C alanine substitution analogs; C). Zone B alanine substitution analogs; D) Zone B truncated analogs.

Table 7. Effects of ACTH(1-24) analogs on rtMC2R activation.

Analog		EC <sub>50</sub> ± SEM (10 <sup>-11</sup> M)	P value
Wild-type	hACTH(1-24)	3.3 ± 1.4	
Zone A	A4	N/A	
	AFRW	77 ± 20*	0.01
	HARW	163 ± 54.4*	0.02
	HFAW	4614 ± 1869*	0.03
	HFRA	2250 ± 1400*	0.03
Zone B	A10-14	1242 ± 161**	0.0008
	A10/14	222 ± 56*	0.01
	KP	27.8 ± 12.3	0.06
	P12	8.1 ± 2.0	0.06
	ACTH(1-22)	N/A	
	ACTH(1-21)	N/A	
Zone C	A5	N/A	
	AARRP	2030 ± 489*	0.007
	KKAAA	3668 ± 778**	0.0046

EC<sub>50</sub> values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (\*:P<0.05, \*\*:P<0.005). NA, No activation



## CHAPTER FIVE

### Site-directed Mutagenesis of TM4, EC2, TM5 in Rainbow Trout MC2R

When ancestral teleosts diverged from ancestral cartilaginous fishes over 400 million years ago, their melanocortin-2 receptor acquired two distinct features that were retained in MC2Rs in amphibians (Liang et al., 2011) and mammals (Cone, 2006): MRAP1 dependence and melanocortin ligand selectivity (Reinick et al., 2012). While these common features are shared by teleosts and tetrapods, during the co-evolution of MC2R, MRAP1 and ACTH, subtle variations could have arose. Take as an example a comparison of the MC2R circuit in humans and the rainbow trout. First examining the ligands, 92% of amino acid residues in the sequence of rainbow trout ACTH(1-24) are identical to human ACTH(1-24). However, the primary sequence identity of melanocortin 2 receptor of these two species drops to 47% (Figure 24).

Analogs of ACTH(1-24) in the three zones had adverse effects on the activation of both human and rainbow trout receptors, and there were subtle differences in the ways that zone A, B and C analogs interacted with these receptors. From the mutant hMC2R experiment discussed in Chapter Three, we have identified six significant sites within TM4/EC2/TM5 region which was responsible for the proposed binding domain for KKRRP motif. In order to find out whether rtMC2R use similar positions or residues as

hMC2R for the interaction with ACTH(1-24), we designed mutant constructs of rtMC2R with alanine substitutions within the same region as the hMC2R mutant study. Since TM4/EC2/TM5 region (22 positions) of rtMC2R contains three neutral, non-polar alanine residues (A156, A160 and A164), we eventually made 19 mutant rtMC2R for receptor activation evaluation using cAMP luciferase reporter assay. For these experiments the wild-type and single alanine mutant constructs of rtMC2R were co-expressed in CHO cells with zfMRAP1 as described in Chapter Four. Luminescence data for receptor activation levels were analyzed as described in Chapter Three.

## **Results**

### **Effects of single alanine substitution in rtMC2R for ACTH signaling**

As performed the comparative experiments for hMC2R mutants study, the corresponding mutated regions of rtMC2R are V153-A160 in TM4, EC2 (F161-I169), and F170-F175 in TM5. A total of 19 mutant rtMC2Rs were individually co-expressed with zfMRAP1 in CHO cells, and receptor functions were examined by cAMP responsive reporter assay. These mutant receptors included 5 mutants at TM4, 8 mutants at EC2, and 6 mutants at TM5. As summarized in Table 8, of 19 residues we examined, 11 residues did not affect ACTH(1-24)-mediated response curve when replaced with alanine. In other words, there were 8 mutant receptors (V157, M158, V159, F161, K168, F171, I172, and F175) that displayed significant decrease in ACTH(1-24)-mediated responses as compared with wild type rtMC2R ( $P < 0.05$ ), whereas in the same region

examined in hMC2R, we only identified 6 significant positions (See Chapter Three, Table 7).

EC<sub>50</sub> value of ACTH(1-24) on wild type rtMC2R was  $(4.6 \pm 0.7) \times 10^{-11}$  M, which was the positive control for all mutant rtMC2Rs. Compared with wild type rtMC2R, three successive positions in TM4 mutants, V157, M158, and V159, had significant negative effects on ACTH dose response curve (Figure 25). The EC<sub>50</sub> values of these mutants were  $(1.9 \pm 0.6) \times 10^{-10}$  M,  $(1.5 \pm 0.3) \times 10^{-10}$  M, and  $(5.1 \pm 1.2) \times 10^{-9}$  M, which were approximately 4-fold, 3-fold, and 110-fold of the wild type hMC2R EC<sub>50</sub> value ( $P=0.04$ ,  $0.03$ , and  $0.01$ , respectively) (Table 8). Note that there was only one significant residue identified from TM4 mutants of hMC2R. Albeit the amino acid residue was threonine (T164) in human receptor instead of valine (V157) in rainbow trout MC2R, they are located at the same position of TM4, indicating a critical role of this position in both human and rainbow trout ACTH/MC2R interaction and receptor signaling.

From eight EC2 mutant receptors, we identified two residues that displayed significant reduction in ACTH(1-24) responses (Figure 26): F161 with a EC<sub>50</sub> of  $(2.2 \pm 0.7) \times 10^{-10}$  M ( $P=0.045$ ) and K169 with a EC<sub>50</sub> of  $(8.2 \pm 0.2) \times 10^{-11}$  M ( $P=0.02$ ) (Table 8). Mutation at these residues induced a 5-fold change in EC<sub>50</sub> of F161 and a 2-fold increase in EC<sub>50</sub> of K169. Compared with the three significant positions (F168, S169 and H170) identified from hMC2R EC2 mutants, position F161 was conserved and also important in rainbow trout and human receptor (F168), while position K169 was significant for ACTH interaction with rtMC2R but not for hMC2R.

Three out of six TM5 mutants greatly diminished ACTH(1-24) responses as compared with wild type rtMC2R (Figure 27A-B), including F171( $EC_{50} = (2.1 \pm 0.8) \times 10^{-9} M$ ), I172 ( $EC_{50} = (2.6 \pm 0.8) \times 10^{-10} M$ ) and F175( $EC_{50} = (4.6 \pm 2.0) \times 10^{-9} M$ ) (Table 8).  $EC_{50}$  values of these mutants were approximately 47-fold, 6-fold, and 100-fold of wild type rtMC2R  $EC_{50}$  respectively ( $P = 0.03, 0.03, 0.04$ ). In addition, position F171 (which is F178 in hMC2R) is a conserved residue in MC2Rs but different with all other MCRs. Mutation on this position greatly altered ACTH(1-24) response in both hMC2R and rtMC2R. These observations indicated that this position is involved in the ligand selectivity of MC2Rs.

Further, we replaced seven significant residues (V157, M158, V159, F161, F171, I172, and F175) with alanine residues in one mutant rtMC2R. The substitutions caused this mutant receptor to lose ACTH(1-24) response completely (Figure 27C). In order to rule out the possible defects on receptor trafficking that might be introduced by alanine substitutions, cell surface ELISA will be performed on rtMC2R mutants in the future.

## Discussion

As discussed in Chapter Three, within TM4/EC2/TM5 region (G162-P183) of human MC2R, we identified six positions that were involved in ACTH(1-24) signaling but not the trafficking of receptors, including T164, F168, S169, H170, F178, and L181. The corresponding 22 amino acid residues in rainbow trout MC2R are from V153 to F175, and we found 8 residues that significantly decreased ACTH(1-24) responses,

which are V157, M158, V159, F161, K168, F171, I172, and F175. Since rtMC2R has a shorter N-terminus than hMC2R, the same position we examined in these two receptors was numbered differently. Therefore, we marked all 14 significant residues with the degrees of EC<sub>50</sub> fold change on TM4/EC2/TM5 sequence alignment diagram for a better comparison (Figure 28). As compared with the EC<sub>50</sub> of wild type MC2R, residues that had significant larger EC<sub>50</sub> were shaded in red in this diagram, suggesting that ACTH(1-24) response were substantially diminished in these mutants. The sequence alignment clearly revealed that three significant sites that were involved in hMC2R/ACTH interaction were important for rtMC2R signaling as well. These residues are T164, F168 and F178 in hMC2R, and V157, F161, and F171 in rtMC2R. At the site T164/V157 of TM4, albeit the threonine in hMC2R was replaced by a valine in rtMC2R, these two amino acids are structurally very similar to each other. The other two positions are both phenylalanine residues F168/F161 and F178/F171, which are located at the beginning of EC2 and TM5. In addition, these phenylalanine residues are both conserved in human, frog, and bony fish MC2Rs but distinct with all other melanocortin receptors (Figure 15). Collectively, these observations indicated that position T164/V157, F168/F161, and F178/F171 are involved in the interacting sites for MC2R to bind ACTH(1-24), specifically on the KKRRP motif of this peptide.

In addition, majority of these significant residues were different between these two receptors, reflecting that the tertiary structure of TM4/EC2/TM5 in these receptor may interact with KKRRP motif in different ways. First, three significant hMC2R residues (S169, H170 and L181) turned out to be unimportant for rtMC2R. Note that

S169 and H170 together with F168 are the first three amino acids in hMC2R EC2. These residues accounts for 33% of entire EC2, and 50% of significant residues identified in hMC2R TM/EC2/TM5 domains. Hence, it appears that the extracellular loop 2 of hMC2R plays a dominant role in terms of the function of this region. Second, newly identified positions from rtMC2R that did not appear in hMC2R mutants were M158, V159, K168, I172, and F175. Putting all 8 significant sites in rtMC2R together, there are three successive positions in TM4, three relatively close positions in TM5, and two discrete positions in EC2. 6 out of 8 positions are present in TM4 and TM5, suggesting the predominant domains for rtMC2R interacting with ACTH(1-24) are TM4 and TM5 instead of EC2.

Based on the fold-change in the  $EC_{50}$  values of mutants as compared with wild type MC2R, these sites were further sorted into three categories (As shown in Figure 28): 1)  $EC_{50}$  larger than 100-fold of wild type MC2R, including F178 of hMC2R, V159 and F175 of rtMC2R (Positions labeled with \*); 2)  $EC_{50}$  larger than 10-fold of wild type MC2R, including H170 of hMC2R and F171 of rtMC2R (Positions labeled with °); and 3)  $EC_{50}$  smaller than 10-fold of wild type MC2R. In this figure, 5 out of 14 residues caused the  $EC_{50}$  values of mutants to increase 10-fold than the wild type control. Note that position F178/F171 in TM5 was the only one position that is identical in these two receptors, restating the importance of this phenylalanine residue. Meanwhile, non-conserved positions in these 5 residues are H170 in EC2 of hMC2R EC2, and V159 and F175 in TM4 and TM5 of rtMC2R. Hence, in addition to the same phenylalanine at TM5, hMC2R and rtMC2R actually use different domains, which are EC2 or TM4/TM5.

In conclusion, the comparative analysis revealed that position T164/V157 in TM4, F168/F161 in EC2, and F178/F171 in TM5 are responsible for the interaction of ACTH(1-24) KKRRP motif, and the importance of these positions are consistent for hMC2R and rtMC2R. However, these receptors have slightly different preference within the overall interaction site. hMC2R uses EC2 as the predominant domain with three successive significant residues, whereas rtMC2R mainly relies on its TM4 and TM5 regions.

```

      N-terminal      [-----TM1-----]      IC1      [-----
hMC2R MKHIINSYENINNTARNNSDCPRVVLPEEIFFTTISIVGVLENLIVLLAVFKNKNLQAPMYFFICS
rMC2R M-----NDVSALPSNHTDCQVVKVPHLVFLVLGMVSLSENLLVVVAVVRNKNLHSPMYMFICS

      -----TM2-----]      EC1      [-----TM3-----]
hMC2R LAISDMLGSLYKILENILIILRNMGYLKPRGSFETTADDIIDSFLVLSLLGSIFSLSVIAADRYI
rMC2R LATFNTISSLSKTTWETLMMEFSDVGQLDSRGDSVRRVDDIIDALLCMSFIGCICSFLAIAVDYRV

      IC2      [-----TM4-----]      EC2      [-----TM5-----
hMC2R TIFHALRYHSIVTMRRTVVVLTVIWTFCTGTGITMVIFSHHVPTVITFTSLFPLMLVFILCLYVH
rMC2R TIFHALRYHNIMTTRRAAALAGIWALCGVAGVMVAFCDATVIKIFFIVLFLISLLLLILFLYVH

      -]      IC      [-----TM6-----]      EC3
hMC2R MFLARSHTRKISTLP-----RANMKGAITLTILLGVFIFCWAPFVLHVLLMTFCSPSNPYCACYM
rMC2R MFLARSHARKIAALPGSAMPHRSLRGALTTLTMLFGVFVVCWAPFFLHLLLLMVCVENPYCECYR

      [-----TM7-----]      C-terminal
hMC2R SLFQVNGMLIMCNAVIDPFIYAFRSPELRDAFKKMIFCSRYW
rMC2R SLFQLNLVLLMSHAVVDPAIYAFRSAELRNTERKMLFCSDSPLCYKVKALFH

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Figure 24. Human and rainbow trout MC2R sequence alignment. Positions shaded in black indicate identical sites in these receptors. h: human; r: rainbow trout (*Oncorhynchus mykiss*).



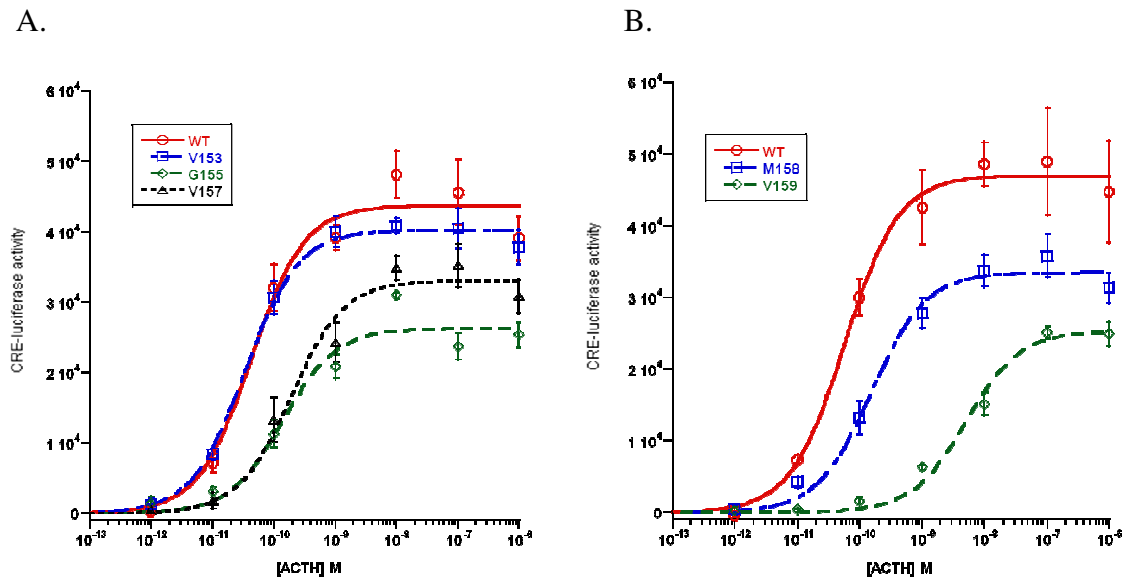


Figure 25. ACTH(1-24) dose response curves of TM4 mutant rtMC2Rs in the presence of zfMRAP1.

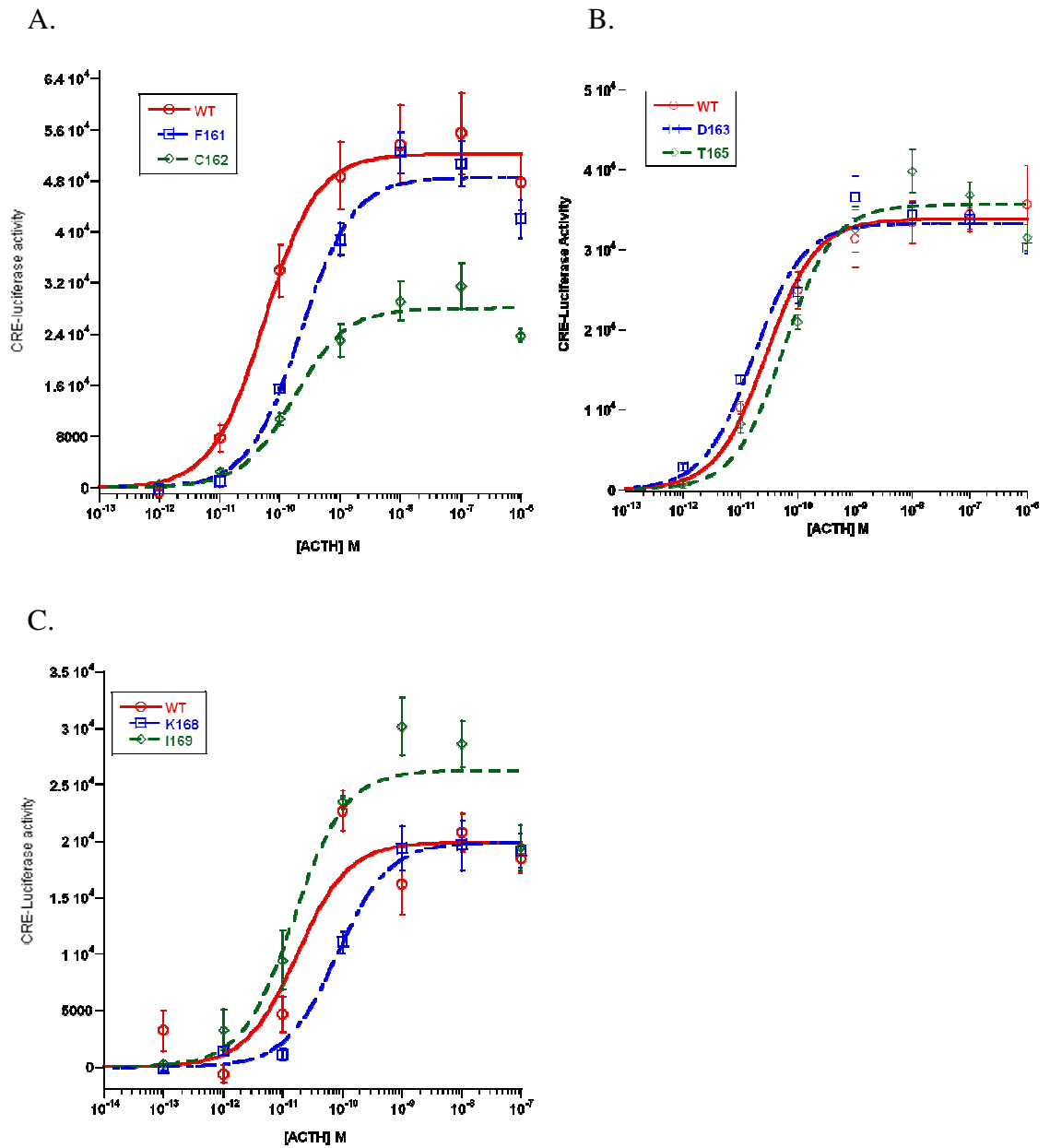


Figure 26. ACTH(1-24) dose response curves of EC2 mutant rtMC2Rs in the presence of zfMRAP1.

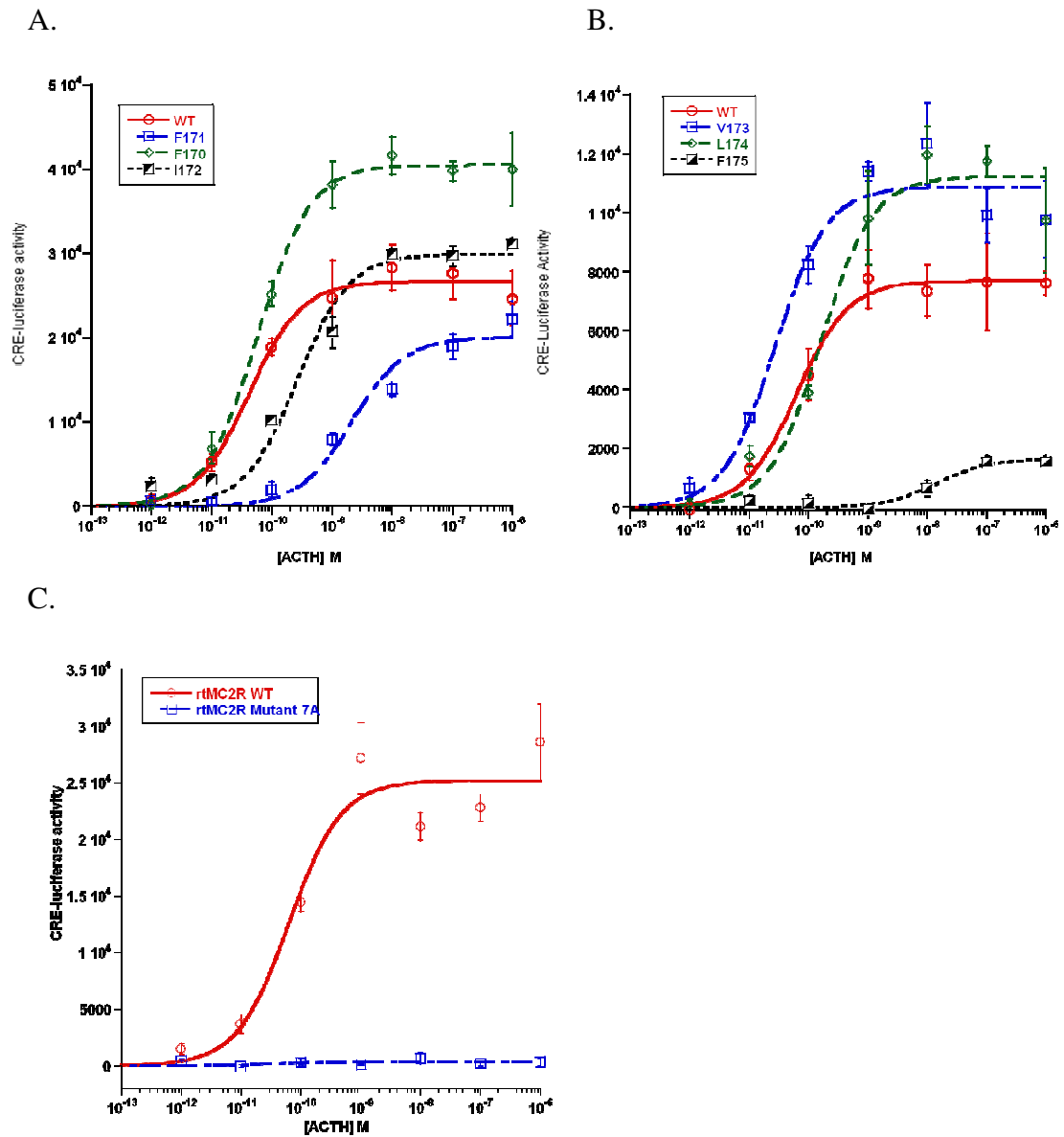


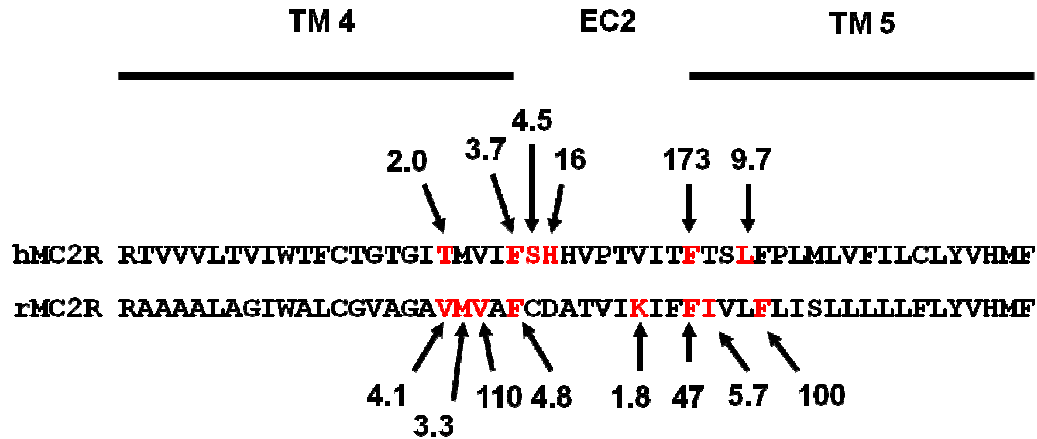
Figure 27. A-B) ACTH(1-24) dose response curves of TM5 mutant rtMC2Rs in the presence of zfMRAP1. C) rtMC2R 7A mutant ACTH(1-24) activation curve.

Table 8. EC<sub>50</sub> values of 19 mutant rtMC2R with single alanine substitution.

Domain	Alanine Substitution	EC <sub>50</sub> ± SEM (10 <sup>-11</sup> M)	P Values
WT	N/A	4.6 ± 0.7	N/A
TM4	V153	3.4 ± 0.46	0.29
	G155	14 ± 6.0	0.1
	V157	19 ± 5.9*	0.037
	M158	15 ± 3.4*	0.027
	V159	510 ± 124*	0.007
EC2	F161	22 ± 7.3*	0.045
	C162	17 ± 6.4	0.077
	D163	1.7 ± 0.59	0.098
	T165	5.8 ± 2.1	0.12
	V166	5.6 ± 2.0	0.089
	I167	2.4 ± 1.1	0.42
	K168	8.2 ± 1.5*	0.02
	I169	1.5 ± 0.9	0.43
TM5	F170	5.8 ± 0.52	0.1
	F171	214 ± 84*	0.03
	I172	26 ± 8.2*	0.027
	V173	3.9 ± 0.68	0.19
	L174	16 ± 5.6	0.081
	F175	460 ± 200*	0.043
Septuple Mutation	V157, M158, V159, F161, F171, I172, F175 (7A)	N/A	

EC<sub>50</sub> values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (\*:P<0.05, \*\*:P<0.005). N/A: No activation. Position A154, A156, A160 and A164 were not tested.

A.



B.

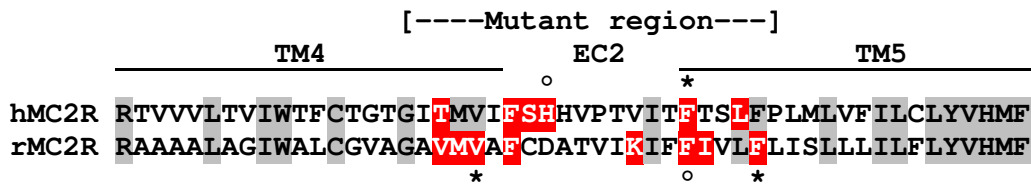


Figure 28. Positions (Red) that significantly affected ACTH signaling in human and rainbow trout MC2R TM4, EC2 and TM5 domains. A) Fold increase of mutant  $EC_{50}$  values as compared with wild type MC2R. B) \*: residues that had an  $EC_{50}$  over 100-fold of wild type MC2R; °: residues that had an  $EC_{50}$  over 10-fold of wild type MC2R; Positions shaded in gray indicate identical sites of these receptors. h: human; r: rainbow trout (*Oncorhynchus mykiss*).

## CONCLUSION

The melanocortin-2 receptor has two functional activation features which are distinct from the other melanocortin receptors (e.g., MC1R, MC3R, MC4R, MC5R): MRAP1 dependency and ligand selectivity for ACTH (Hinkle and Sebag 2009; Webb and Clark, 2010). These features of MC2R had appeared since the ancestral ray-finned fishes diverged from ancestral cartilaginous fishes over 400 million years ago (Reinick et al., 2011). This study focused on the important sites in both ACTH(1-24) and MC2R that are responsible for receptor activation, and whether this pattern has changed between mammal (human) and bony fish (rainbow trout) MC2Rs.

In the sequence of melanocortin peptides, the HFRW motif is required in order to activate melanocortin receptors (Figure 2B). Previous studies on human MC4R identified ten residues located in the TM2, TM3, TM6 and TM7 regions that are involved in the binding of the HFRW motif (Pogozheva et al, 2005). In human MC1R, MC3R, MC4R and MC5R 90% of these residues are identical, and as a result all of these receptors can be activated by ACTH or any type of MSHs. In MC2Rs, 70% of these residues are conserved in human, frog and rainbow trout MC2R orthologs as well (Figure 24). As a result, the HFRW motif binding site for MC2R in human and rainbow trout should be at the same positions (i.e., TM2, TM3, TM6, and TM7). Zone A analog stimulation results

(see Chapter 1) indicated that the HFRW motif was critical for both hMC4R and MC2R (human and rainbow trout) with a subtle difference predicted at the tertiary structure level for the two receptors (Table 3 and Table 7). The tryptophan (W<sup>9</sup>) was the most important position in the HFRW motif for the activation of both hMC2R and hMC4R, whereas in rainbow trout MC2R, the arginine (R<sup>8</sup>) seems to be more important than tryptophan (W<sup>9</sup>). In addition to the requirement for the HFRW motif, the KKRRP motif was also essential for MC2R activation, especially with respect to the rainbow trout receptor (Table 7). In addition, residues R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> are more important than K<sup>15</sup>K<sup>16</sup> in both hMC2R and rtMC2R (table 3 and Table 7). Finally, the sequence between the HFRW motif and the KKRRP motif is GKPVG. Replacement of individual residues of this region did not adversely affect receptor activation of both hMC2R and rtMC2R. Nevertheless, this region should be important, because incubation of receptor-transfected CHO cells with two peptides each containing HFRW motif and KKRRP motif respectively (i.e.,  $\alpha$ -MSH and the KKRRP analog), was unable to activate hMC2R (Figure 11B). This controversy was resolved by testing two truncated analog ACTH(1-22) and ACTH(1-21) which were also unable to activate hMC2R and rtMC2R as well (Table 3 and Table 7). Collectively, it is now clear that the sequence of the HFRW motif and the KKRRP motif in ACTH(1-24) can activate hMC2R and rtMC2R as a result of the sequence length in between these two motifs (i.e., GKPVG) that properly orients the ligand to its binding sites in the receptor.

After conducting studies on the features of ACTH(1-24), this thesis then addressed questions relating to the mechanism for activation of MC2R. Although both HFRW and KKRRP motif are required in MC2R activation, they play different roles in

the binding of ACTH(1-24) to MC2R. Schwyzer (1977) had proposed that the KKRRP motif in ACTH played a critical role in the binding of the ligand to the receptor. As indicated in Chapter 2, ACTH(15-24), the KKRRP motif analog, significantly reduced ligand binding affinity, whereas analog A4, the HRFW motif analog, did not show any effect on the binding curve (Table 3). Furthermore, a short peptide (KKRRPVKVYP) only containing the last ten amino acid residues in ACTH(1-24) was able to bind to hMC2R to the same degree as full length ACTH(1-24) (Figure 12D). These observations led to the discovery of an ACTH(1-24) antagonist (KKRRPVKVYP) that specifically targets hMC2R instead of other melanocortin receptors. In our *in vitro* system, the application of this antagonist can block ACTH(1-24) activation completely at the concentration of 100nM, and it can reduce ACTH(1-24) activation by 35% when applied at concentration of 10nM (Figure 12C). These observations lend support to the hypothesis presented in Figure 10 that ACTH first binds to MC2R at the KKRRP motif, and then the HFRW motif in the ligand is positioned into HFRW binding site on the receptor because of the “bridging” action of the GKPVG motif in the ligand.

The studies on the ACTH antagonist set the stage for the characterization of the KKRRP binding site on the receptor (Chapter 3). The KKRRP binding site in MC2R appears to be the predominant region that is not only defining its ligand selectivity, but also initiating the binding of ACTH(1-24). The 3-dimensional model for MCRs (Figure 14) predicts that the transmembrane domains for HFRW binding site (TM2, TM3, TM6 and TM7) are at close proximity and form the space to allow HFRW motif to sit inside. Based on that orientation, it would follow that the KKRRP motif of ACTH should be



positioned within the region close to TM4, TM5 and EC2 (Figure 14). In order to test this hypothesis, we made single-alanine mutant receptors at this region in both human and rainbow trout. Although the sequence identity between hMC2R and rtMC2R for this region is only 32% (Figure 28), the importance of certain positions in TM4, EC2, and TM5 for the activation of receptor became apparent. It appears that TM5 was important for both receptors (F178 hMC2R/F171 F175 rtMC2R), whereas EC2 (H170) of hMC2R and TM4 (V159) of rtMC2R were also involved in receptor activation (Figure 28). Of all the residues in this region that adversely affected the  $EC_{50}$  by a factor of 5 fold or greater, only F178 in hMC2R and F175 in rtMC2R are at the same relative position in their respective receptor. Clearly the 3-dimensional structure of this binding pocket must be the feature that human receptor and the rainbow trout receptor share in common.

In the next step in this research it would be informative to use ACTH(15-24) to probe the proposed KKRRP binding site. For these experiments, the iodinated competitive inhibitor (KKRRPVKVYP) would be made in order to support the conclusion that these sites in MC2R are involved in the binding of KKRRP motif of ACTH. When applied to the mutant receptors, the prediction would be that the competitive inhibitor should cause a decline in the binding curves as compared to the wild-type receptor. Furthermore, 3D structure modeling analysis of MC2R should be done in the future to obtain the detailed description of receptor/ligand relationship.

## WORKS CITED

- Butler A.A. 2006. The melanocortin system and energy balance. *Peptides*, 27, pp. 281–290.
- Cerdá-Reverter JM, Agulleiro MJ, R RG, Sánchez E, Ceinos R, Rotllant J. 2011. Fish melanocortin system. *Eur J Pharmacol.* 660(1):53-60.
- Chan L.F., Metherell L.A., Clark A.J. 2011. Effects of melanocortins on adrenal gland physiology. *Eur J Pharmacol.* 60(1):171-80.
- Chan LF, Clark AJ, Metherell LA. 2008. Familial glucocorticoid deficiency: advances in the molecular understanding of ACTH action. *Horm Res.* 69(2):75-82.
- Chen M., Aprahamian C.J., Kesterson R.A., Harmon C.M., Yang Y. 2007. Molecular identification of the human melanocortin-2 receptor responsible for ligand binding and signaling. *Biochemistry* 46: 11389-11397.
- Chen W, Kelly MA, Opitz-Araya X, Thomas RE, Low MJ, Cone RD. 1997. Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell*, 91 (6), pp. 789–798.
- Chen, M., Aprahamian, C. J., Celik, A., Georgeson, K. E., Garvey, W. T., Harmon, C. M., and Yang, Y. 2006. Molecular characterization of human melanocortin-3 receptor ligand-receptor interaction, *Biochemistry* 45, 1128-1137.
- Chepurny, O.G., Holz, G.G., 2007. A novel cyclic adenosine monophosphate responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein coupled receptor drug discovery efforts. *J. Biomol. Screen.* 12, 740–746.
- Chhajlani V., Wikberg J.E. 1992. Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. *FEBS Lett.*, 309 (3), pp. 417–420.
- Chung T.T., Webb T.R., Chan L.F., Cooray S.N., Metherell L.A., King P.J., Chapple J.P., Clark A.J. 2008. The majority of ACTH receptor (MC2R) mutations found in Familial Glucocorticoid Deficiency type 1 lead to defective trafficking of the receptor to the cell surface. *Journal of Clinical Endocrinology and Metabolism.* 93(12): 4948-4954.
- Ciruela F, Vilardaga JP, Fernández-Dueñas V. 2010. Lighting up multiprotein complexes: lessons from GPCR oligomerization. *Trends Biotechnol.* 28(8):407-15.

- Cole MA, Kim PJ, Kalman BA, Spencer RL. 2000. Dexamethasone suppression of corticosteroid secretion: evaluation of the site of action by receptor measures and functional studies. *Psychoneuroendocrinology* 25:151–167.
- Cone, R.D. 2006. Studies on the physiological functions of the melanocortin system. *Endocr Rev* 27, 736–749.
- Cooray S.N., Chan L., Webb T.R., Metherell L., Clark A.J. 2009. Accessory proteins are vital for the functional expression of certain G protein-coupled receptors. *Mol Cell Endocrinol.* 5;300(1-2):17-24.
- Cooray S.N., Clark A.J. 2011. Melanocortin receptors and their accessory proteins. *Mol Cell Endocrinol.* 15;331(2):215-21.
- Costa JL, Bui S, Reed P, Does RM, Hochgeschwender U, Brennan MB. 2004. Mutational Analysis of Evolutionarily Conserved ACTH Residues. *Gen. Comp. Endocrinol.* 136 :12-16.
- Cushing H. 1932. The basophil adenomas of the pituitary body and their clinical manifestations (pituitary basophilism). *Bull Johns Hopkins Hosp* 50:137-195.
- Devi LA. 2005. *The G-Protein-Coupled Receptors Handbook*. Humana Press, New Jersey.
- Does R.M. 2009. Adrenocorticotrophic hormone, melanocyte-stimulating hormone, and the melanocortin receptors: revisiting the work of Robert Schwyzler: a thirty-year retrospective. *Ann N Y Acad Sci.* 1163:93-100.
- Does RM, Lecaude S. 2005. Trends in the evolution of the proopiomelanocortin gene. *Gen. Comp. Endocrinol.* 142:81-93.
- Eberle A, Schwyzler R. 1975. Hormone-receptor interactions. Demonstration of the two message sequences (active sites) in  $\alpha$ -melanocortopin. *Helv. Chim. Acta* 58:1528-1535.
- Eipper B, Mains R. 1980. Structure and Biosynthesis of Pro-Adrenocorticotropin/Endorphin and Related Peptides. *Endocrine Reviews* 1:1-27.
- Entwistle ML, Hann LE, Sullivan DA, Tatro JB. 1990. Characterization of functional melanotropin receptors in lacrimal glands of the rat Peptides. 11 (3), pp. 477–483.
- Forti FL, Dias MH, Armelin HA. 2006. ACTH receptor: ectopic expression, activity and signaling. *Mol Cell Biochem.* 293(1-2):147-60.
- Fridmanis, D., Petrovska, R., Kalnina, I., Slaidina, M., Peculis, R., Schioth, H.B., Klovins, J. 2010. Identification of domains responsible for specific membrane transport and ligand specificity of the ACTH receptor (MC2R). *321(2):175-83.*

Gantz I., Konda Y., Tashiro T., Shimoto Y., Miwa H., Munzert G., Watson S.J., DelValle J., Yamada T. 1993. Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *J. Biol. Chem.* 268: 15174–15179.

Gantz, I., Fong, T.M. 2003. The melanocortin system. *Am J Physiol Endocrinol Metab* 284, E468–E474.

García-Borrón JC, Sánchez-Laorden BL, Jiménez-Cervantes C. 2005. Melanocortin-1 receptor structure and functional regulation. *Pigment Cell Res.* 18(6):393-410.

Haskell-Luevano, C., Sawyer, T. K., Trumpp-Kallmeyer, S., Bikker, J. A., Humblet, C., Gantz, I., and Hruby, V. J. 1996. Three-dimensional molecular models of the hMC1R melanocortin receptor: Complexes with melanotropin peptide agonists, *Drug Des. DiscoVery* 14, 197-211.

Hinkle PM, Sebag JA. 2009. Structure and function of the melanocortin2 receptor accessory protein (MRAP). *Mol Cell Endocrinol.* 300(1-2):25-31.

Hoogduijn M.J., McGurk S., Smit N.P., Nibbering P.H., Ancans J., van der Laarse A., Thody A.J. 2002. Ligand-dependent activation of the melanocortin 5 receptor: cAMP production and ryanodine receptor-dependent elevations of  $[Ca^{2+}]_i$  *Biochem. Biophys. Res. Commun.*, 290, pp. 844–850.

Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F. 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell.* 88(1):131-41.

Jacobson L, Sapolsky R. 1991. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr Rev* 12:118–134.

Keller-Wood ME, Dallman MF. 1984. Corticosteroid inhibition of ACTH secretion. *Endocr Rev* 5:1–24.

Konda, Y., Gantz, I., DelValle, J., Shimoto, Y., Miwa, H., and Yamada, T. 1994. Interaction of dual intracellular signaling pathways activated by the melanocortin-3 receptor. *J. Biol. Chem.* 269, 13162 – 13166.

Leatherland JF, Li M, Barkataki S., 2010. Stressors, glucocorticoids and ovarian function in teleosts. *J Fish Biol.* 76(1):86-111.

Lee JH, Lim SK, Huh SH, Lee D, Lee W. 1998. Solution structures of the melanocyte-stimulating hormones by two-dimensional NMR spectroscopy and dynamical simulated-annealing calculations. *Eur J Biochem.* 257(1):31-40.

- Liang L, Sebag JA, Egelston L, Serasinghe MN, Veo K, Reinick C, Angleson J, Hinkle PM, Dore RM. 2011. Functional expression of frog and rainbow trout melanocortin 2 receptors using heterologous MRAP1s. *Gen Comp Endocrinol.* 174(1):5-14.
- Lightman SL, Conway-Campbell BL. 2010. The crucial role of pulsatile activity of the HPA axis for continuous dynamic equilibration. *Nat Rev Neurosci.* 11(10):710-8.
- Liu, J., Rone, M. P. & Papadopoulos, V. 2006. Protein-protein interactions mediate mitochondrial cholesterol transport and steroid biosynthesis. *Journal of Biological Chemistry* 281, 38879–38893.
- Lundin LG. 1993. Evolution of the vertebrate genome as reflected in paralogous chromosomal regions in man and the house mouse. *Genomics* 16: 1-19.
- Marks N, Stern F, Kastin AJ. 1976. Biodegradation of alpha-MSH and derived peptides by rat brain extracts, and by rat and human serum. *Brain Res Bull.* 1(6):591-3.
- Martin W.J., MacIntyre D.E. 2004. Melanocortin receptors and erectile function. *Eur. Urol.*, 45 (6), pp. 706–713
- Metherell, L.A., Chapple, J.P., Cooray, S., David, A., Becker, C., Ruschendorf, F., Naville, D., Begeot, M., Khoo, B., Nurnberg, P., Huebner, A., Cheetham, M.E., Clark, A.J. 2005. Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. *Nat. Genet.* 37 (2), 166–170.
- Mommsen, T. P., Vijayan, M. M. & Moon, T. W. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* 9, 211–268.
- Mormede P, Foury A, Barat P, Corcuff JB, Terenina E, Marissal-Arvy N, Moisan MP. 2011. Molecular genetics of hypothalamic-pituitary-adrenal axis activity and function. *Ann N Y Acad Sci.* 1220:127-36.
- Mountjoy K.G., Wild J.M. 1998. Melanocortin-4 receptor mRNA expression in the developing autonomic and central nervous systems. *Brain Res. Dev. Brain Res.*, 107 (2), pp. 309–314
- Nakanishi S, Inoue A, Kita T, Nakamura M, Chung ACY, Cohen SN, Numa S. 1979. Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* 278: 423-427.
- Ohno S, Wolf U, Atkins NB. 1968. Evolution from fish to mammals by gene duplication. *Hereditas* 59:169–187.

Pecoraro N, Dallman MF, Warne JP, Ginsberg AB, Laugero KD, la Fleur SE, Houshyar H, Gomez F, Bhargava A, Akana SF. 2006. From Malthus to motive: how the HPA axis engineers the phenotype, yoking needs to wants. *Prog Neurobiol* 79:247–340.

Pogozheva, I.D., Chai, B.X., Lomize, A.L., Fong, T.M., Weinberg, D.H., Nargund, R.P., Mulholland, M.W., Grants, I., and Mosberg, H.I. 2005. Interactions of human melanocortin 4 receptors with nonpeptide and peptide agonists. *Biochemistry* 44, 11329-11341.

Rached M, El Mourabit H, Buronfosse A, Blondet A, Naville D, Begeot M, Penhoat A. 2005. Expression of the human melanocortin-2 receptor in different eukaryotic cells. *Peptides*. Oct;26(10):1842-7.

Roselli-Reh fuss L., Mountjoy K.G., Robbins L.S., Mortrud M.T., Low M.J., Tatro J.B., Entwistle M.L., Simerly R.B., Cone R.D. 1993. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc. Natl. Acad. Sci. U.S.A.*, 90 (19), pp. 8856–8860.

Roy, S., Perron, B., Gallo-Payet, N. 2010. Role of asparagine-linked glycosylation in cell surface expression and function of the human adrenocorticotropin receptor (melanocortin 2 receptor) in 293/FRT cells. *Endocrinology*. 151, 660–670.

Schiöth HB, Haitina T, Ling MK, Ringholm A, Fredriksson R, Cerda-Reverter JM, Klovins J. 2005. Evolutionary conservation of the structural pharmacological and genomic characteristics of the melanocortin receptor subtypes. *Peptides* 26:1886-1900.

Schwyzler R. 1977. ACTH: a short introductory review, *Ann. NY Acad. Sci.* 297: 3-26.

Seelig S, Lindley BD, Sayers G. 1975. A new approach to the structure-activity relationship for ACTH analogs using isolated adrenal cortex cells, In: *Methods in Enzymol.* 39: 347-59 (J. G. Hardman, B. W. O'malley, eds). Academic Press, New York  
Slominski A, Ermak G, Mihm M. 1996. ACTH receptor, CYP11A1, CYP17 and CYP21A2 genes are expressed in skin. *J. Clin. Endocrinol. Metab.*, 81 (7), pp. 2746–2749.

Thomas, P. 2008. The endocrine system. In *The Toxicology of Fishes* (Di Giulio, R. T. & Hinton, D. E., eds), pp. 457–488. New York, NY: Taylor & Francis.

Tritos NA, Biller BM. 2012. Advances in medical therapies for Cushing's syndrome. *Discov Med.* 13(69):171-9.

Västermark A, Schiöth HB. 2011. The early origin of melanocortin receptors, agouti-related peptide, agouti signaling peptide, and melanocortin receptor-accessory proteins,

- with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus. *Eur J Pharmacol.* 660(1):61-9.
- Veo K, Reinick C, Liang L, Moser E, Angleson JK, Dores RM. 2011. Observations on the ligand selectivity of the melanocortin 2 receptor. *Gen Comp Endocrinol.* 172(1):3-9.
- Wachira, S.J., Hughes-Darden, C.A., Taylor, C.V., Ochillo, R., and Robinson, T.J. 2003. Evidence for the interaction of protein kinase C and melanocortin 3-receptor signaling pathways. *Neuropeptides* 37, 201 – 210.
- Webb TR, Clark AJ. 2010. Minireview: the melanocortin 2 receptor accessory proteins. *Mol Endocrinol.* 24(3):475-84.
- Yang Y. 2011. Structure, function and regulation of the melanocortin receptors. *Eur J Pharmacol.* 660(1):125-30.
- Yang, Y. K., Fong, T. M., Dickinson, C. J., Mao, C., Li, J. Y., Tota, M. R., Mosley, R., van der Ploeg, L. H., and Gantz, I. 2000. Molecular determinants of ligand binding to the human melanocortin-4 receptor, *Biochemistry* 39, 14900-14911.
- Yang, Y., Dickinson, C., Haskell-Luevano, C., and Gantz, I. 1997. Molecular basis for the interaction of [Nle<sup>4</sup>,D-Phe<sup>7</sup>]melanocyte stimulating hormone with the human melanocortin-1 receptor, *J. Biol. Chem.* 272, 23000-23010.
- Ying J, Kövér KE, Gu X, Han G, Trivedi DB, Kavarana MJ, Hruby VJ. 2003. Solution structures of cyclic melanocortin agonists and antagonists by NMR. *Biopolymers.* 71(6):696-716.
- Zhu CC, Cook LB, Hinkle PM. 2002. Dimerization and phosphorylation of thyrotropin-releasing hormone receptors are modulated by agonist stimulation. *J Biol Chem* 277:28228–28237.

## **APPENDIX 1**





## Minireview

## Observations on the ligand selectivity of the melanocortin 2 receptor

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## ARTICLE INFO

## Article history:

Available online 8 April 2011

## Keywords:

Melanocortin receptors

MC2R

Activation of a GPCR

## ABSTRACT

The melanocortin 2 receptor (MC2R) is unique in terms of ligand selectivity and *in vitro* expression in mammalian cell lines as compared to the other four mammalian MCRs. It is well established that ACTH is the only melanocortin ligand that can activate the ACTH receptor (i.e., melanocortin 2 receptor). Recent studies have provided new insights into the presence of a common binding site for the HFRW motif common to all melanocortin ligands. However, the activation of the melanocortin 2 receptor requires an additional amino acid motif that is only found in the sequence of ACTH. This mini-review will focus on these two topics and provide a phylogenetic perspective on the evolution of MC2R ligand selectivity.

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## 1. Introduction

Although the pituitary hormones  $\alpha$ -MSH and ACTH were characterized over 50 years ago [25,26], the biosynthetic relationship between these two polypeptides as well as their common mode of action would not become apparent until the initial characterization of the mRNA that codes for the proprotein, POMC [32], and the discovery of the melanocortin receptor gene family (see for review [7]). The initial enigma with respect to these two polypeptides was their common amino acid sequence.  $\alpha$ -MSH is the first thirteen amino acids in the sequence of ACTH(1–39) for every vertebrate species that has been studied [12] with the exception of the lampreys [43]. Furthermore, ACTH(1–39) is a major end-product in the corticotrophic cells of the anterior pituitary, whereas,  $\alpha$ -MSH is a major end-product in the melanotropic cells of the intermediate pituitary [4]. The explanation for the differential distribution of these two polypeptides was resolved when it was discovered that these polypeptides are derived from the prohormone precursor, POMC [32]. Although ACTH(1–39) is an end-product in the corticotrophic cells of the anterior pituitary, in the melanotropic cells of the intermediate pituitary this same polypeptide functions as a biosynthetic intermediate and gives rise to  $\alpha$ -MSH (for review see [13]). The subsequent discovery of the proprotein convertases, PC1/3 and PC2 (for review see [39]), provided the mechanism to explain the tissue specific posttranslational processing of POMC in the anterior and intermediate lobes of the pituitary [10,28,46].

Viewing the POMC system from a physiological perspective, ACTH(1–39) serves as a chemical signal in the hypothalamus/pituitary/adrenal-interrenal axis (HPA/I), whereas,  $\alpha$ -MSH plays no physiological role in this circuit [4,6]. By comparison,  $\alpha$ -MSH ( $\alpha$ -

melanocyte-stimulating hormone) regulates physiological color change through the activation of pigment granule translocation in dermal chromatophores [6,16]. More recent studies indicate that  $\alpha$ -MSH can also function as a neuromodulator in the hypothalamus and plays a role in the regulation of appetite/feeding behavior in mammals and perhaps in all vertebrates [6,35]. Given the level of primary sequence identity between  $\alpha$ -MSH and ACTH(1–39), were these polypeptides acting through the same receptor on target cells or were there distinct “ACTH” and “ $\alpha$ -MSH” receptors? The characterization of the melanocortin receptors (MCR) from the genome of the mouse [7] and the subsequent characterization of these GPCR genes from all the major classes of vertebrates resolved this question [40], but also raised new issues.

## 2. Phylogeny of MCRs

The phylogenetic distribution of MCRs in various vertebrate classes is summarized in Fig. 1. The discovery of two melanocortin-related receptors in the lamprey genome [17] coupled with the presence of five MCR genes in the genome of tetrapods points to the role of genome duplication events in the radiation of this GPCR gene family during chordate evolution. Since it is assumed that an ancestral MCR gene originated in some early chordate lineage, the two genome duplication events that are predicted to have occurred during chordate evolution [21,27,33] should account for the presence of four paralogous melanocortin receptor genes in gnathostomes, yet five paralogous genes are present in tetrapods [7] and some bony fish genomes [40]. In order to explain these observations, one scenario proposed that the initial outcome of the two rounds of genome duplication events gave rise to MC1R, MC3R, MC4R genes and a proposed proto-MC5R/MC2R gene [5]. Synteny studies [22] would appear to support the assumption that a local gene duplication of a putative proto-MC5R/MC2R gene gave

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mouse MC2R gene [31], attempts to study this unique ligand/receptor relationship were hampered by another unique feature of the bony vertebrate MC2R orthologs.

#### 4. MC2R and the MRAPs

Although the MC1R, MC3R, MC4R, and MC5R genes can be functionally expressed in a variety of cell lines, the MC2R gene could only be functionally expressed in cell lines derived from the adrenal cortex [14,30,36,38] or cell lines, like the rat INS-1  $\beta$ -cells [2], which like mouse and human  $\beta$ -cells express the *mc2r* gene. Recent studies have revealed that cells which are capable of functionally expressing MC2R also co-express the melanocortin receptor accessory protein (*mrp*) gene. In mammals and perhaps many non-mammalian vertebrate species there are two paralogous *mrp* genes (*mrp1* and *mrp2*). The mammalian MRAP gene products have been extensively studied and the salient features of these two proteins have been recently reviewed [20,45]. The properties of these proteins can be summarized as follows. The MRAP gene products are single chain polypeptides with a single transmembrane spanning domain. Following synthesis at the RER, MRAP1 monomers form a homodimer in which the two polypeptide chains are oriented in an antiparallel manner. MRAP2 also forms a homodimer (20,44). When the *mc2r* gene is transfected into cells that do not endogenously express the *mrp1* or the *mrp2* genes, the trafficking of newly synthesized MC2R from the ER to the plasma membrane will not occur. In addition, in cells transfected with the *mc2r* gene and the *mrp2* gene, trafficking of the newly synthesized MC2R to the plasma membrane will occur, but the MC2R on the plasma membrane cannot be subsequently activated by ACTH. However, in cells that are transfected with the *mc2r* gene and the *mrp1* gene, not only does trafficking of the newly synthesized MC2R to the plasma membrane occur, these receptors can also be activated by ACTH. Collectively, these observations indicate that MRAP1 and MRAP2 are intracellular chaperones that facilitate the movement of MC2R to the plasma membrane. In addition it appears that MRAP1 must be in contact with MC2R at the plasma membrane in order for the receptor to be in the proper conformation to bind ACTH and be activated by this ligand. Finally a recent study indicates that at the plasma membrane MC2R is present as a homodimer. The MC2R homodimer is combined with two MRAP1 homodimers to make a hexameric complex. The hexameric complex, then, is the functional "ACTH" receptor on the plasma membrane of the transfected cells [8]. In summary, the functional expression of MC2R in cells in culture requires the co-expression of a *mc2r* gene construct and a *mrp1* gene construct. Is the interaction between MRAP1 and MC2R an ancestral feature of the *mc2r* gene? It would appear so. Recent studies on the activation of zebrafish MC2R indicate that a zebrafish *mrp1* construct must be co-transfected into HEK 293 cells in order for the zfMC2 receptor to be activated by ACTH [1].

#### 5. Binding and activation sites on MCRs

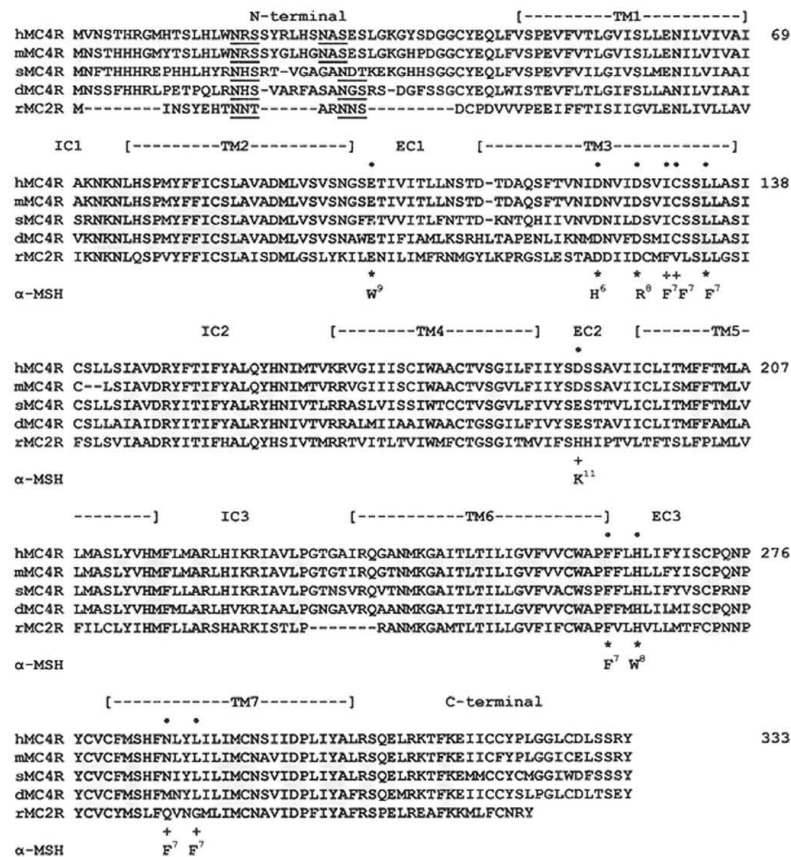
Given the distinctive features of MC2R in terms of ligand activation and functional expression, what properties does MC2R share in common with MC1R, MC3R, MC4R, and MC5R? Since all five melanocortin receptors can be activated by ligands that have the amino acid motif, HFRW, it would be reasonable to assume that all vertebrate melanocortin receptors share a common HFRW binding site. That hypothesis was tested by Pogozheva et al. [34] using a complicated, yet elegant strategy to model the putative HFRW binding site in the human MC4 receptor. The model identified TM 2, 3, 6, and 7 as the most likely regions to form a binding site for the HFRW motif of mammalian  $\alpha$ -MSH. The intriguing feature

of the model was the identification of a site that could accommodate the  $\beta$ -hairpin turn in the HFRW motif [19]. The model was tested by performing single alanine substitutions to selected residues in TM 2, 3, 6, and 7 regions that could potentially have ionic interactions or form H-bonds with residues in the HFRW motif of  $\alpha$ -MSH. The single-alanine mutant forms of human MC4R were transfected into HEK 293 cells and the production of cAMP in these cells was compared to HEK 293 cells transfected with the wild-type form of human MC4R. The results of those experiments are summarized in Fig. 2. In the sequence of human MC4R there are 11 amino acids distributed between TM2, TM3, EC2, TM6, and TM7 that are involved in the  $\alpha$ -MSH binding site. It is interesting that the majority of these sites are predicted to make contact with the phenylalanine residue in the HFRW motif of  $\alpha$ -MSH. Ten of these residues are present in the sequences of *Squalus acanthias* MC4R and *Silurana tropicalis* MC4R (Fig. 2). Since several of these same residues are also found in the melanocortin receptors cloned from the lamprey genome [5], this binding pocket must have emerged early in the evolution of the vertebrates.

For at least MC1R, MC3R, MC4R, and MC5R, the HFRW binding site is also the activation site for these receptors [41]. Since the phenylalanine and the arginine residues in the HFRW motif form the reverse beta-turn in this portion of the melanocortin core sequence, would alanine substitution of either of these residues eliminate activation of the MC4 receptor? To test this possibility, GT1-7 cells [29] that endogenously express the mouse *mc4r* gene were used [42]. As shown in Fig. 3A, these cells can be stimulated by either  $\alpha$ -MSH or ACTH(1–24). The GT1-7 cells were then challenged with analogs of ACTH(1–24) in which the HFRW motif was either: (a) replaced with four alanine residues (A4 analog); (b) the F<sup>7</sup> residue was replaced with an alanine residue (HARW analog); or (c) the R<sup>8</sup> residue was replaced by an alanine residue (HFAW analog). As shown in Fig. 3B, at concentrations of either 10<sup>−6</sup> or 10<sup>−9</sup> M none of these analogs of ACTH(1–24) activated the MC4 receptor on the GT1-7 cells, whereas cells incubated with ACTH(1–24) were clearly activated at both concentrations. Since the HFRW motif in a melanocortin ligand is responsible for both binding to and activating the receptor [41], the results presented in Fig. 3B would appear to indicate that none of the alanine-substituted ACTH(1–24) analogs may have bound to the MC4 receptor; hence no activation. However, when the A4, HARW, and HFAW analogs, at a concentration of 10<sup>−6</sup> M were individually co-incubated with ACTH(1–24) at concentrations of either 10<sup>−9</sup> or 10<sup>−10</sup> M, there was a significant drop in activation as compared to cells stimulated with either concentration of ACTH(1–24) without the analogs present (Fig. 3C). These results indicate that for lower concentrations of ACTH(1–24) the alanine-substituted analogs may be acting as competitive inhibitors which would imply that residues either N-terminal or C-terminal to the HFRW motif are also playing a role in the positioning of the melanocortin ligand at the HFRW binding site on the receptor.

Given these observations for the MC4R, and it is assumed that MC1R, MC3R, and MC5R would respond in a similar manner, what are the implications for the melanocortin 2 receptor? As indicated in Fig. 2 the rat MC2 receptor has six of the eleven residues found in the HFRW binding site for MC4Rs. However as already noted, mammalian MC2Rs, and perhaps all bony vertebrate MC2Rs, can only be activated by ACTH(1–24), but not by  $\alpha$ -MSH [N-acetyl-ACTH(1–13)amide]. The key, then, to understanding the activation of an MC2 receptor would appear to lie in residues 14 to 24 in the sequence of ACTH(1–24). As reviewed by Schwyzler [41], the shortest analog of ACTH(1–24) that still retains some level of biological activity is ACTH(1–16), and conversely analogs longer than ACTH(1–16) are progressively stronger agonists. Costa et al. [9] conducted a series of studies that focused on residues in the motif K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> in the sequence of hACTH(1–24). Alanine substitu-



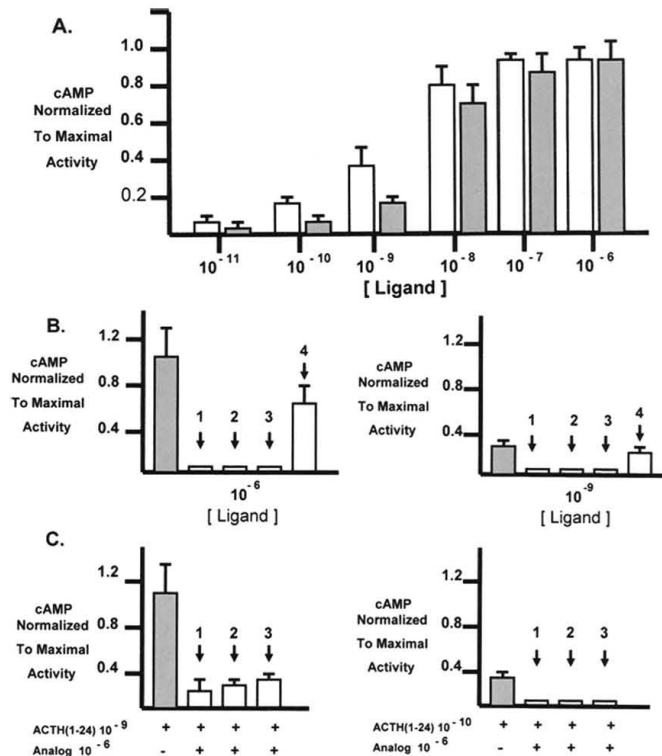


**Fig. 2.** Comparison of MC4R and MC2R amino acid sequences. The amino acid sequences of MC4R orthologs from human (h: AA092061.1), mouse (m: AAI16958.1), *Silurana tropicalis* (s: <http://genome.jgi-psf.org/Xent4/Xent4.home.html>), and dogfish (d: AA039833.1) were aligned as described previously [11] and compared to the sequence of rat MC2R (r: NP\_001093961.1). In this data set 34% of the positions are identical and these positions are shaded in gray. The residues in human MC4R that are predicted to form the HFRW binding site are marked with a • [34]. The residues in the predicted HFRW site in the other MCR sequences which are identical to the corresponding positions in hMC4R are marked with a \*. Finally, the residues that are variable in the predicted HFRW bind site are marked with +. The predicted correspondence between residues in the HFRW motif of  $\alpha$ -MSH and residues in the predicted HFRW binding site on the MCRs [34] is presented.

tions at any of these sites lowered the biological activity of the ACTH(1–24) analog with varying degrees of efficacy. Collectively, the experiments using C-terminally modified analogs of ACTH(1–24) point to the KKRRP motif in mammalian ACTH(1–24) as important for the activation of MC2R. If this conclusion is correct, the prediction would be that replacement of the KKRRP motif in mammalian ACTH(1–24) with alanines (A5 analog) should produce an analog with a greatly diminished capacity for activating MC2R. As observed in Fig. 3B, the A5 analog of ACTH(1–24) can activate the MC4 receptor on GT1–7 cells. Hence, these substitutions do not adversely affect the conformation of the ACTH(1–24) with respect to interaction with MC4R at the HFRW binding site of this receptor. However, when rat INS-1 cells, which endogenously express the MC2 receptor [2], were incubated with the A5 analog there was a significant drop in the activation of the MC2 receptor as compared to stimulation by ACTH(1–24) (Fig. 4). This result would support the importance of the KKRRP motif in mammalian

ACTH(1–24) for the activation of the MC2 receptor and also suggests that residues at positions 20–24 may play some role in the activation of the receptor.

The model that emerges to explain the activation of MC2R by ACTH(1–24) appears to be dependent on the presence of two sites in the receptor: an HFRW binding and activation site and a KKRRP binding site. The former site is not accessible to MSH-sized ligands, whereas binding of ACTH(1–24) to the latter site would appear to properly position ACTH(1–24) so that the N-terminal of the ligand can make contact with the HFRW binding site on MC2R to activate the receptor. Intrinsic to this two step model is the interaction between MC2R and MRAP1 at the plasma membrane. Since zebrafish MC2R has the same requirements for activation as mammalian MC2R (presence of MRAP1 and activation by ACTH(1–24), but not by  $\alpha$ -MSH [1]) this mechanism must have been present in the common ancestor to the ancestral bony fishes and the ancestral lobe-finned fishes. These two requirements (MRAP1 dependence



**Fig. 3.** Activation of MC4R on GT1-7 cells by  $\alpha$ -MSH and ACTH(1-24). GT1-7 (GnRH neurons) cells endogenously express the mouse MC4R [29,42]. The cells were maintained as described in [29,42]. GT1-7 cells were seeded at a density of  $6 \times 10^5$  cells/well in a 6-well plate. (A) After 48 h, GT1-7 cells were stimulated with either NDP-MSH (white histograms) or hACTH(1-24) (gray histograms) (New England Peptide) at doses ranging from  $10^{-11}$  to  $10^{-6}$  M. The ligand was delivered in "stimulating solution" that had the composition: 140 mM NaCl, 2.0 mM KCl, 1.0 mM  $MgCl_2$ , 5.0 mM  $CaCl_2$ , 10 mM HEPES, 10 mM D-glucose, 0.1% BSA and supplemented with 1 mM IBMX (pH adjusted to 7.2). Cells were then lysed using 0.1 M HCl. The cAMP levels in the samples were measured using the Direct cAMP EIA kit (Enzo Life Sciences Inc.) and analyzed on a Bio-Tek Synergy HT plate reader. Data are expressed as mean  $\pm$  SEM ( $n = 3$  wells of GT1-7 cells). In the negative control wells an equal number of GT1-7 cells were incubated with the stimulation solution without ligand. Data were normalized to the maximal response for the saturating doses of NDP-MSH. (B) In separate wells GT1-7 cells at a density of  $6 \times 10^5$  cells/well were stimulated with either hACTH(1-24) (gray histogram), the A4 analog [SYSMEAAAAGKPVGKRRPVKVYP] (1), the HAFW analog [SYSMEHARWGWGKRRRPVKVYP] (2), the HFAW analog [SYSMEHFAWGWGKRRRPVKVYP] (3), or the A5 analog [SYSMEHFRWGWGKPVGAAAAVKVYP] (4) at concentrations of either  $10^{-6}$  or  $10^{-9}$  M as described for (A). After a 15 min stimulation period, the cells were lysed and cAMP levels in the samples were measured using the Direct cAMP EIA kit (Enzo Life Sciences Inc.). Data are expressed as mean  $\pm$  SEM ( $n = 3$  wells of GT1-7 cells). Data were normalized to the maximal response for the saturating doses of ACTH(1-24). Analogs 1, 2, and 3 did not activate the MC4 receptor ( $P < 0.05$ ). (C) In separate wells GT1-7 cells were incubated for 15 min with either ACTH(1-24) alone at a concentration of  $10^{-9}$  M (gray histogram) or co-incubated with ACTH(1-24) at concentration of  $10^{-9}$  M and analogs 1, 2, and 3 at concentration of  $10^{-6}$  M. In a separate experiment, GT1-7 cells were incubated for 15 min with either ACTH(1-24) alone at a concentration of  $10^{-10}$  M (gray histogram) or co-incubated with ACTH(1-24) at concentration of  $10^{-10}$  M and analogs 1, 2, and 3 at concentration of  $10^{-6}$  M. Each experiment was conducted as described in the legend for (A). Data were normalized to the maximal response for ACTH(1-24) at  $10^{-9}$ . In the negative control wells an equal number of GT1-7 cells were incubated with the stimulation solution without ligand. Analogs 1, 2, and 3 significantly inhibited the activation of the MC4 receptor in the presence of  $10^{-9}$  and  $10^{-10}$  M ACTH(1-24) ( $P < 0.05$ ).

and strict ligand selectivity) are the features that define a functional melanocortin 2 receptor, and set MC2R apart from the other members of the MCR gene family.

## 6. Conclusions and future questions

The functional expression of a melanocortin 2 receptor requires interaction with MRAP1 in order for activation of MC2R to be possible. Is there a common site on vertebrate MC2Rs for interaction with MRAP1? Can a mammalian MRAP1 facilitate the activation of a fish MC2R?

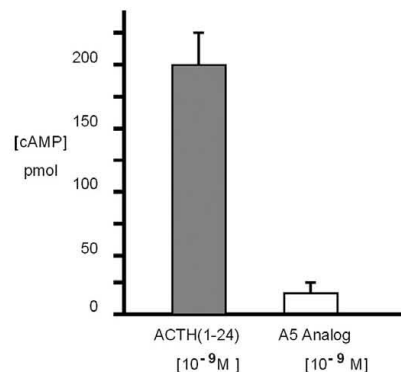
When did the MRAP1/MC2R interaction appear in vertebrate evolution? Perhaps this question can be addressed by studies on cartilaginous fishes. Studies on *Squalus acanthias* yielded orthologs of MC3R and MC4R that could be functionally expressed in HEK

293 cells, however an ortholog of MC5R could not be expressed in these cells and as a result the ligand selectivity properties of this MCR have not been determined [23,37]. Does *Squalus acanthias* MC5R have a requirement for MRAP, and can this MCR be selectively activated by ACTH, but not by the shark MSH ligands?

The functional definition of a melanocortin 2 receptor is that it is a MCR that can only be activated by ACTH, but not by any of the MSHs. When did MC2R evolve a conformation that would accommodate ACTH as a ligand, but exclude  $\alpha$ -MSH? A recent phylogenetic study has identified an apparent ortholog of MC2R in the genome of the elephant shark, *Callorhynchus milii* [44]. Will the putative *C. milii* MC2R have a requirement for MRAP and what are the ligand selectivity properties of this GPCR?

Finally, as predicted by Schwyzner [41], positions 16 through 19 in ACTH(1-39) are important for the activation of MC2R. Can the





**Fig. 4.** Stimulation of INS1 cells by ACTH(1–24) of the A5 analog of ACTH(1–24). INS1 cells endogenously express the rat *mc2r* gene [2]. To determine whether the A5 analog of ACTH(1–24) [SYSMEHFRWGKPVGAAAAVKVYP] can activate MC2R, INS1 cells were seeded at a density of  $6 \times 10^5$  cells/well. After 48 h, cells were stimulated with either ACTH(1–24) [ $1 \times 10^{-9}$ ] or ACTH analog A5 [ $1 \times 10^{-9}$ ] for 15 min at 37 °C as described in the legend to Fig. 3. Following lysis of the cells, cAMP levels were measured using the Direct cAMP EIA kit (Enzo Life Sciences Inc.). Data are expressed as mean  $\pm$  SEM ( $n = 3$  wells of INS1 cells). At this concentration the stimulation of INS1 cells by the A5 ACTH analog was significantly lower ( $P < 0.050$ ) as compared to stimulation of INS1 cells by ACTH(1–24). In the negative control wells an equal number of INS1 cells were incubated with the stimulation solution without ligand.

binding of C-terminal analogs like ACTH(15–24) activate MC2R alone or can the binding of C-terminal analogs of ACTH make the receptor amenable to activation by  $\alpha$ -MSH? Finally, where in MC2R is the binding site for the ACTH(15–19) motif of ACTH?

#### Acknowledgments

We thank Professor Pamela Mellon (U.C. San Diego) for the gift of the GT1-7 cells. This research was supported by NSF Grant IOB 0516958.

#### References

- [1] M.J. Aguilero, S. Roy, E. Sánchez, S. Puchol, N. Gallo-Payet, J.M. Cerda-Reverter, Role of melanocortin receptor accessory proteins in the function of zebrafish melanocortin receptor type 2, *Mol. Cell Endocrinol.* 320 (2010) 145–152.
- [2] H.T. Al-Majed, P.M. Jones, S.J. Persaud, D. Sugden, G.C. Huang, S. Amiel, B.J. Whitehouse, ACTH stimulates insulin secretion from MIN6 cells and primary mouse and human islets of Langerhans, *J. Endocrinol.* 180 (2004) 155–166.
- [3] M. Aluru, M.M. Vijayan, Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout, *Endocrinology* 149 (2008) 4577–4588.
- [4] B. Baker, The evolution of ACTH, MSH and LPH: structure, function and development, in: E.J.W. Barrington (Ed.), *Hormone Evolution*, 2 (1980) Academic Press, New York, 643–722.
- [5] A. Baron, K. Veo, J. Angleson, R.M. Does, Modeling the Evolution of the MC2R and MC5R genes: Studies on the Cartilaginous Fish, *Heterodontus francisci*, *Gen. Comp. Endocrinol.* 161 (2009) 13–19.
- [6] J.M. Cerda-Reverter, M.A. Aguilero, R. Guillot, E. Sanchez, R. Ceinos, J. Rotlant, Fish melanocortin system. *European J. Pharmacology* doi:10.1016/j.ejphar.2010.10.108.
- [7] R.D. Cone, Studies on the Physiological Studies of the Melanocortin System, *Endocr. Rev.* 27 (2006) 736–749.
- [8] S.N. Cooray, T.-T. Chung, K. Mazhar, L. Szidonya, A.J.L. Clark, Bioluminescence resonance energy transfer reveals the adrenocorticotropin (ACTH)-induced conformational change of the activated ACTH receptor complex in living cells, *Endocrinology* 152 (2011) 495–502.
- [9] J.L. Costa, S. Bui, P. Reed, R.M. Does, U. Hochgeschwender, M.B. Brennan, Mutational Analysis of Evolutionarily Conserved ACTH Residues, *Gen. Comp. Endocrinol.* 136 (2004) 12–16.
- [10] R. Day, M.K.-H. Schafer, S.J. Watson, M. Chretien, N.G. Seidah, Distribution and regulation of the prohormone convertases PC1 and PC2 in the rat pituitary, *Mol. Endocrinol.* 6 (1992) 485–497.

- [11] R.M. Does, D.A. Rubin, T.W. Quinn, Mini-review: is it possible to construct phylogenetic trees using polypeptide hormone sequences, *Gen. Comp. Endocrinol.* 103 (1996) 1–12.
- [12] R.M. Does, S. Lecaude, Trends in the evolution of the proopiomelanocortin gene, *Gen. Comp. Endocrinol.* 142 (2005) 81–93.
- [13] B.A. Eipper, R.E. Mains, Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides, *Endocr. Rev.* 1 (1980) 1–27.
- [14] F.L. Forti, M.H.S. Dias, H.A. Armelin, H.A. ACTH, Receptor: ectopic expression activity and signaling, *Mol. Cell. Biochem.* 293 (2006) 147–160.
- [15] R. Fredriksson, M.C. Lagerstrom, L.G. Lundin, H.B. Schiöth, The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints, *Mol. Pharmacol.* 63 (2003) 1256–1272.
- [16] I.I. Geschwind, Chemistry of the melanocyte-stimulating hormone, in: G. Della Porta, O. Mühnbock (Eds.), *Structure and Control of Melanocytes*, Springer Verlag, Berlin, pp. 28–44.
- [17] T. Haitina, J. Klovins, A. Takahashi, M. Löwgren, A. Ringholm, J. Enberg, H. Kawauchi, E.T. Larson, R. Fredriksson, H.B. Schiöth, Functional characterization of two melanocortin (MC) receptors in lamprey showing orthology to the MC1 and MC4 receptor subtypes, *BMC Evol. Biol.* 7 (2007) 101–115.
- [18] T. Haitina, A. Takahashi, L. Holmen, J. Enberg, H.B. Schiöth, Further evidence for ancient role of ACTH peptides at melanocortin (MC) receptors; pharmacology of dogfish and lamprey peptides at dogfish MC receptors, *Peptides* 28 (2007) 798–805.
- [19] E.M. Haslach, J.W. Schaub, C. Haskell-Luevano, Beta-turn secondary structure and melanocortin ligands, *Bioorg. Med. Chem.* 17 (2009) 952–958.
- [20] P.M. Hinkle, J.A. Sebag, Structure and function of the melanocortin 2 receptor accessory protein, *Mol. Cell. Endocrinol.* 300 (2009) 25–31.
- [21] P.W. Holland, J. Garcia-Fernandez, N.A. Williams, A. Sidow, Gene duplications and the origins of vertebrate development. *Development (Supplement)* (1994) 125–133.
- [22] J. Klovins, T. Haitina, D. Fridmanis, Z. Kilianova, I. Kapa, R. Fredriksson, N. Gallo-Payet, H.B. Schiöth, The melanocortin system in fugu: determination of POMC/AGRP/MCR gene repertoire and synteny As well as pharmacology and anatomical distribution of the MCRs, *Mol. Biol. Evol.* 21 (2004) 563–579.
- [23] J. Klovins, T. Haitina, A. Ringholm, M. Löwgren, D. Fridmanis, M. Slaidina, et al., Cloning of two melanocortin (MC) receptors in spiny dogfish, *Eur. J. Biochem.* 271 (2004) 4320–4331.
- [24] Y. Kobayashi, H. Chiba, T. Yamanome, H.B. Schiöth, A. Takahashi, Melanocortin receptor subtypes in interrenal cells and corticotropin activity of  $\alpha$ -melanocyte-stimulating hormones in barfin flounder, *Verasper moseri*, *Gen. Comp. Endocrinol.* 170 (2011) 558–568.
- [25] T.H. Lee, A.B. Lerner, Isolation of melanocyte-stimulating hormone from hog pituitary, *J. Biol. Chem.* 221 (1956) 943–959.
- [26] C.H. Li, I.I. Geschwind, R.D. Cole, L.D. Raake, J.I. Harris, J.S. Dixon, Amino-acid sequence of alpha-corticotropin, *Nature* 176 (1955) 687–689.
- [27] L.G. Lundin, Evolution of the vertebrate genome as reflected in paralogous chromosomal regions in man and the house mouse, *Genomics* 16 (1993) 1–19.
- [28] M. Marcinkiewicz, R. Day, N.G. Seidah, M. Chretien, Ontogeny of the prohormone convertases PC1 and PC2 in the mouse hypophysis and their colocalization with corticotropin and  $\alpha$ -melanocortin, *Proc. Natl. Acad. Sci. USA* 91 (1993) 5784–5787.
- [29] P.L. Mellon, J.J. Windle, P.C. Goldsmith, C.A. Padula, J.L. Roberts, R.I. Weiner, Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis, *Neuron* 5 (1990) 1–10.
- [30] L.A. Metherell, J.P. Chapple, S. Cooray, A. David, C. Becker, F. Ruschendorf, D. Naville, M. Begeot, B. Khoo, P. Nurnberg, A. Huebner, M.E. Cheethan, A.J. Clark, Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2, *Nat. Genet.* 37 (2005) 166–170.
- [31] K.G. Mountjoy, L.S. Robbins, M.T. Mortrud, R.D. Cone, The cloning of a family of genes that encode the melanocortin receptors, *Science* 257 (1992) 1248–1251.
- [32] S. Nakanishi, A. Inoue, T. Kita, M. Nakamura, A.C.Y. Chung, S.N. Cohen, S. Numa, Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor, *Nature* 278 (1979) 423–427.
- [33] S. Ohno, U. Wolf, N.B. Atkins, Evolution from fish to mammals by gene duplication, *Heredity* 59 (1968) 169–187.
- [34] I.D. Pogozheva, B.-X. Chai, A.L. Lomize, T.M. Fong, D.H. Weinberg, R.P. Nargund, M.W. Mulholland, I. Grantz, H.I. Mosberg, Interactions of human melanocortin 4 receptors with nonpeptide and peptide agonists, *Biochemistry* 44 (2005) 11329–11341.
- [35] L.E. Prichard, A.V. Turnbull, A. White, Review: pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signaling and obesity, *J. Endocrinol.* 172 (2002) 411–421.
- [36] M. Rachel, H. El Mourabit, A. Buronfosse, A. Blondet, D. Naville, M. Begeot, A. Penhoat, Expression of the human melanocortin-2 receptor in different eukaryotic cells, *Peptides* 26 (2005) 1842–1847.
- [37] A. Ringholm, J. Klovins, R. Fredriksson, N. Poliakova, E.T. Larson, J.P. Kukkonen, D. Larhammar, H.B. Schiöth, Presence of melanocortin (MC4) receptor in spiny dogfish suggests an ancient vertebrate origin of central melanocortin system, *Eur. J. Biochem.* 270 (2003) 213–221.
- [38] S. Roy, M. Rachel, N. Gallo-Payet, Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms  $\alpha$  and  $\beta$  in isogenic human embryonic kidney 293 cells, *Mol. Endocrinol.* 21 (2007) 1656–1669.

- [39] N.G. Seidah, M. Chrétien, Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides, *Brain Res.* 848 (1999) 45–62.
- [40] H.B. Schiöth, T. Haitina, M.K. Ling, A. Ringbom, R. Fredriksson, J.M. Cerdá-Reverter, J. Klovins, Evolutionary conservation of the structural, pharmacological and genomic characterization of the melanocortin receptors subtypes, *Peptides* 26 (2005) 1886–1900.
- [41] R. Schwyzler, ACTH: a short introductory review, *Ann. NY Acad. Sci.* 297 (1977) 3–26.
- [42] H. Shinyama, H. Mazuzaki, H. Fang, J.S. Flier, Regulation of melanocortin-4 receptor signaling: agonist-mediated desensitization and internalization, *Endocrinology* 144 (2003) 1301–1314.
- [43] A. Takahashi, Y. Amemiya, M. Sarashi, S.A. Sower, H. Kawauchi, Melanotropin and corticotropin are encoded on two distinct genes in the lamprey, The earliest evolved extant vertebrate, *Biochem. Biophys. Res. Commun.* 213 (1995) 490–496.
- [44] A. Vastermark, H.B. Schiöth, The early origin of melanocortin receptors, agouti-related peptides, agouti signaling peptide, and melanocortin receptor accessory proteins with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus. *European Journal Pharm.* doi:10.1016/j.ejphar.2010.10.106.
- [45] T.R. Webb, J.L. Clark, Minireview: the melanocortin 2 receptor accessory proteins, *Mol. Endocrinol.* 24 (2010) 475–484.
- [46] A. Zhou, R.E. Mains, Endoproteolytic processing of proopiomelanocortin and prohormone convertase 1 and 2 in neuroendocrine cells over expressing prohormone convertase 1 and 2, *J. Biol. Chem.* 269 (1994) 17440–17447.

## **APPENDIX 2**



## Functional expression of frog and rainbow trout melanocortin 2 receptors using heterologous MRAP1s

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### ARTICLE INFO

#### Article history:

Received 17 May 2011

Revised 21 July 2011

Accepted 21 July 2011

Available online 9 August 2011

#### Keywords:

Melanocortin 2 receptor

MRAP1

cAMP

*Xenopus tropicalis*

*Oncorhynchus mykiss*

### ABSTRACT

Analysis of the functional expression of the melanocortin 2 receptor (MC2R) from a rather broad spectrum of vertebrates indicates that MC2R is exclusively selective for the ligand, ACTH, and the melanocortin receptor accessory protein 1 (MRAP1) is required for high affinity ACTH binding and activation of MC2R. A phylogenetic analysis of MRAP1 suggested that tetrapod sequences and bony fish sequences may represent two distinct trends in the evolution of the *mrp1* gene. To test this hypothesis, a frog (*Xenopus tropicalis*) MC2R was expressed in CHO cells either in the presence of a tetrapod (mouse) MRAP1 or a bony fish (zebrafish) MRAP1. The response of frog MC2R to different concentrations of human ACTH(1–24) was more robust in the presence of mouse MRAP1 than in the presence of zebrafish MRAP1. Conversely, the cAMP response mediated by the rainbow trout (*Oncorhynchus mykiss*) MC2R was almost twofold higher and occurred at 1000-fold lower ACTH concentration in the presence of zebrafish MRAP1 than in the presence of mouse MRAP1. Collectively, these experiments raise the possibility that at least two distinct trends have emerged in the co-evolution of MC2R/MRAP1 interactions during the radiation of the vertebrates.

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### 1. Introduction

The biosynthesis of transmembrane proteins, such as the G-protein coupled receptors (GPCRs), involves the translation of the GPCR mRNA at the rough endoplasmic reticulum (RER), followed by the trafficking of the GPCR through the endoplasmic reticulum (ER) and golgi, leading to incorporation of the GPCR into the lipid bilayer of a secretory vesicle at the trans golgi. The eventual merging of the secretory vesicle with the plasma membrane will result in the positioning of the GPCR on the plasma membrane [28]. Since this pathway is present in all eukaryotic cells many GPCRs have been functionally expressed in a variety of eukaryotic cells. In the case of the melanocortin receptors (MCRs), GPCRs that can be activated by the melanocortin ligands (i.e., ACTH,  $\alpha$ -MSH,  $\beta$ -MSH, and  $\gamma$ -MSH) [6], MC1R, MC3R, MC4R, and MC5R can be functionally expressed in, for example, HEK293 cells. The heterologous expression of these MCRs in a mammalian cell line has been achieved not only for human and mouse orthologs of these receptors (for review see [6]), but also for the MCR orthologs of two species of bony fish [4,14], and the MCR orthologs of a cartilaginous fish [15,22]. However, there are two exceptions to this generalization. The MC5R

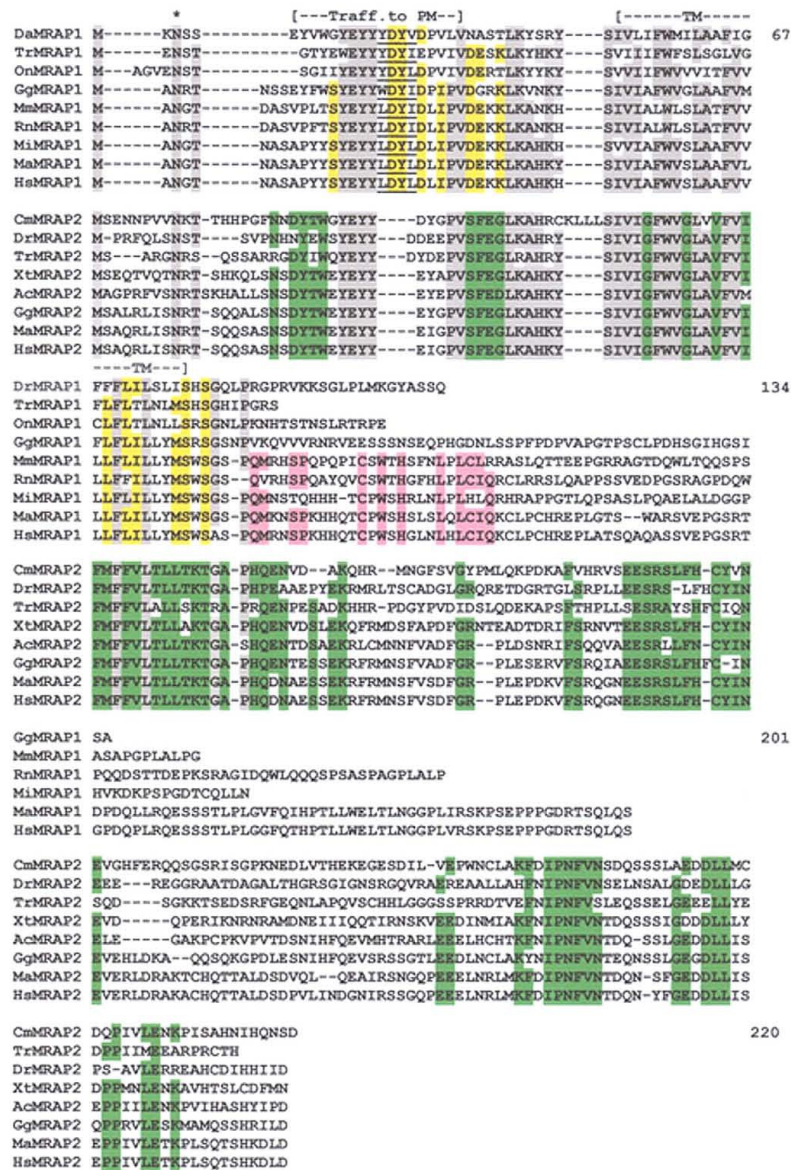
ortholog from the spiny dogfish *Squalus acanthias* has not been successfully expressed in HEK293 cells [15]. In addition, neither the mouse MC2R ortholog [18], or for that matter any vertebrate MC2R ortholog has been functionally expressed in HEK293 cells when the MC2R ortholog alone was transfected into these cells [12,31]. This study will focus on the heterologous expression of MC2R orthologs.

The open reading framing encoding MC2R (ACTH receptor) was initially isolated by screening a human genomic library [18], and a MC2R cDNA construct was subsequently functionally expressed in Cloudman S91 melanoma cells. Later studies found that mammalian MC2R constructs could not be functionally expressed in heterologous cells lines unless those cell lines were derived from the adrenal cortex [9,17,21,24]. In addition, it was observed that a subset of patients exhibiting familial glucocorticoid deficiency type 2 syndrome had functional MC2 receptors, yet were resistant to ACTH treatment [17]. Collectively, these observations led to the discovery that adrenal cortical cells express the *melanocortin receptor accessory protein (mrp)* gene [17]. In the genomes of many vertebrates there are two paralogs *mrp* genes (*mrp1* and *mrp2*) [12,31]. As shown in Fig. 1, MRAP1 and MRAP2 are single chain polypeptides with a single membrane spanning domain. Both paralogs form antiparallel homodimers in the ER [25–27], and this unique feature appears to be associated with the residues located

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**Fig. 1.** Alignment of MRAP1 and MRAP2 amino acid sequences. The MRAP1 amino acid sequences for *Homo sapiens* (Hs), *Macaca mulatta* (Ma), *Microcebus murinus* (Mi), *Rattus norvegicus* (Rn), *Mus musculus* (Mm), *Gallus gallus* (Gg), *Oreochromis niloticus* (On), *Takifugu rubripes* (Tr), and *Danio rerio* (Dr) were aligned with the MRAP2 amino acid sequences from *Homo sapiens* (Hs), *Macaca mulatta* (Ma), *Gallus gallus* (Gg), *Anolis carolinensis* (Ac), *Xenopus tropicalis* (Xt), *Takifugu rubripes* (Tr), *Danio rerio* (Dr), and *Callorhynchus milii* (Cm). Amino acid positions with 70% or greater primary sequence identity in both paralogs are shaded in gray, amino acid positions with 66% or greater primary sequence identity in MRAP1 orthologs only are shaded in yellow, (c) amino acid positions with 80% or better primary sequence identity in mammalian MRAP1 orthologs only are shaded in pink, and amino acid positions with 75% or better primary sequence identity in MRAP2 orthologs only are shaded in green. The N-linked glycosylation site present in all vertebrate MRAPs is marked with a (\*). The domain in the N-terminal region responsible for trafficking MC2R to the plasma membrane is located between positions 21 and 38. The transmembrane domain (TM) is located between positions 54 and 77. LDYL/I motif predicted to be critical for facilitating the activation of MC2R is underlined, as are the corresponding amino acid positions in *G. gallus*, *O. niloticus*, *T. rubripes*, and *D. rerio* MRAP1.

between positions 43 and 55 in Fig. 1. In addition, each paralog has a domain located between positions 21 and 38 to facilitate the trafficking of MC2R from the ER to the plasma membrane [30]. Given

these common features, the homodimers of both MRAP1 and MRAP2 will form a complex with MC2R in the ER and facilitate the trafficking of mammalian MC2Rs to the plasma membrane.

However, only MRAP1/MC2R complexes on the plasma membrane can be efficiently activated by ACTH, whereas the efficacy of ACTH activation is greatly diminished for MRAP2/MC2R complexes [12,31]. Finally a recent study indicates that when a human *mrp1* cDNA construct and a human *mc2r* cDNA construct are co-transfected into HEK293 cells, the functional ACTH receptor is a hexamer composed of a MC2R homodimer and two MRAP1 homodimers [7].

The role of MRAP1 in the trafficking and activation of MC2R is not limited to mammals. A recent study indicates that zebrafish MC2R can be activated only when a zebrafish *mc2r* cDNA construct is co-transfected with a zebrafish *mrp1* construct in HEK293 cells [1]. Collectively these studies, on widely divergent vertebrate species (mammals and a bony fish), would suggest that all vertebrate MC2Rs require MRAP1 for functional expression. It also appears highly likely that all vertebrate MC2Rs can only be activated by ACTH, but not by any of the MSH-related melanocortin ligands [6]. Thus, *in vitro* studies to understand the evolution of the unique ligand selectivity properties of MC2R in the various lineages of bony vertebrates (e.g., ray finned fishes, lobe finned fishes, anamniote tetrapods and amniote tetrapods) require that the MC2R of interest be co-expressed with MRAP1. In the heterologous expression studies that have been cited, an MC2R has been paired with the MRAP1 from the same species or at least from the same class of vertebrates. Is it possible to achieve functional expression of a MC2R by co-expressing that receptor with an MRAP1 from another taxonomic class? If this were possible it would alleviate situations in which the sequence of the MC2R ortholog is known for a species, but the MRAP1 ortholog has not been determined for that species. Two examples are the amphibian *Xenopus (Silurana) tropicalis* (frog) and the teleost *Oncorhynchus mykiss* (rainbow trout). For the former species the sequence of MC2R has been deduced from *X. tropicalis* genome data base (<http://genome.jgi-psf.org/Xentr4/Xentr4.home.html>), but at this time no *mrp1* ortholog has been detected in that data base. For the latter species, the rainbow trout MC2R ortholog has been cloned and sequenced [2], but the sequence of a MRAP1 ortholog has not been determined. The objective of this study was to determine whether the functional expression of the *X. tropicalis* MC2R and the *O. mykiss* MC2R could be achieved by co-transfecting either the frog MC2R or the rainbow trout MC2R with either a mammalian or bony fish MRAP1.

## 2. Materials and methods

### 2.1. MC2R and MRAP constructs

Rainbow trout (*Oncorhynchus mykiss*) [2], Accession No. EU119870, and frog (*Xenopus tropicalis*) MC2R, Accession No. XP002936118.1 were synthesized by GenScript with a V-5 epitope tag and these cDNAs were separately inserted into a pcDNA3.1 vector, respectively. Zebrafish (*Danio rerio*) MRAP1, Accession No. XP001342923.2, was synthesized by GenScript with a FLAG epitope tag and inserted into a pcDNA3.1 vector. The human (h) MC2R cDNA was obtained from the Missouri S&T cDNA Resource Center. The mouse (m) MRAP1 was provided by Dr. Adrian Clark at Queen Mary University (London, United Kingdom).

*Xenopus* MRAP2 was cloned from adrenocortical tissue using the following primers: forward primer contain NheI site, V5 epitope tag sequence and Kozak sequence: 5'-TATATATATAGCTAGCGCCAC-CATGGGTAAGCCTATCCCCAACCCCTCTGCTCGGTCTTGATTCTACTTGT-GAACAACTGTTCAAC-3' and the reverse primer with a MluI site with no stop codon: 3'-TATATATATAACGCGTCATAAAGTCACACA-AACTAGTATGG-5'. The sequence of the amplicon agreed with the published *X. tropicalis* MRAP2 sequence (ENSXETG00000018157). The PCR product was cloned into a pCI-Neo vector containing a 3xFLAG epitope sequence followed by a stop codon in frame with a MluI site.

### 2.2. Cell growth

Most of the experiments were done in transiently transfected CHO cells. The CHO cells were grown at 37° in a humidified 5% CO<sub>2</sub> incubator in DMEM/F12 with 5% fetal calf serum. *X. tropicalis* MC2R was also transiently transfected in an A6 *Xenopus* kidney fibroblast cell line kindly provided by Dr. Jacques Robert at the University of Rochester Medical Center (Rochester, NY). A6 cells were grown at 27 °C in a humidified 5% CO<sub>2</sub> incubator in basic amphibian serum-free medium supplemented with 10% fetal calf serum as described [23].

### 2.3. Immunocytochemical staining of CHO cells expressing MC2R and MRAP

CHO cells were grown in 8-well chamber slides (5 × 10<sup>4</sup> cell/well) for 24 h prior to transfection. The cells were transfected with 1 µg of either the rainbow trout or frog *mc2r* cDNA construct either in the presence or absence of the mouse *mrp1* cDNA construct using lipofectamine 2000 and Opt-Mem. After 24 h post-transfection, the cells were fixed with 4% paraformaldehyde and either permeabilized with 0.3% triton for 10 min (Fig. 7A) or left unpermeabilized (Fig. 7B–D). The primary antibodies were diluted 1:500 in PBS + 1% BSA solution. A mouse monoclonal anti-V5 antibody was used to detect MC2 receptors. A rabbit polyclonal anti-FLAG antibody was used to detect mMRAP1. Cells were incubated with primary antibodies for 1 h at 37 °C. Following a wash step the cells were incubated with secondary antibodies for 45 min at 37 °C. The secondary antibodies were diluted 1:800 in PBS + 1% BSA solution. A donkey anti-mouse antibody linked to Alexa-Fluor488 was used to visualize MC2 receptors (green). A donkey anti-rabbit antibody Alexa-Fluor555 was used to visualize mMRAP1 (red). For colocalization of MC2R and mMRAP1, unpermeabilized cells were incubated with both primary antisera, followed by incubation with both secondary antibodies (Fig. 7C and D). As a control, untransfected CHO cells were stained with the V-5/donkey anti-mouse antisera and no reactions were observed. In addition, CHO cells transfected with the MC2R construct were incubated with secondary antisera, but not primary antisera. Once again no reaction was observed. For all staining experiments, cover slips were mounted onto slides using Vectashield and nuclei were stained with DAPI (blue). Images were acquired using a 100X oil immersion objective (Zeiss Plan-Neofluar) with a fluorescence microscope equipped with a Hamamatsu digital camera. All images were analyzed using SlideBook software.

### 2.4. cAMP and expression assays

For the functional expression of the hMC2 receptor and the frog MC2 receptor a cAMP reporter assay was used. Cells were plated in 96 well dishes and transfected with 40 ng/well total DNA using FugeneHD according to manufacturer's instructions. Transfections typically were performed using 13.3 ng/well of a cAMP-dependent luciferase reporter containing multiple copies of the cAMP response element (CRE) from the rat insulin promoter [5] provided by Dr. George Holz (Syracuse, NY), 21.3 ng/well receptor DNA and 5.3 ng/well MRAP or GFP DNA. After 24 h, the medium was replaced with 40 µl fresh DMEM/F12 containing 0.1% BSA with no additions, 20 µM forskolin or ACTH(1–24) from Phoenix Biochemicals at the concentrations shown. After 5 h, medium was aspirated and One Step Luciferase Assay Reagent (Nanolight Technologies) was added. Luminescence was read on a BioTek platereader. Values shown represent the mean and SEM from triplicate wells in a representative experiment.

Cell surface expression of hMC2 receptors, which contained an extracellular, N-terminal triple HA tag, and total expression of

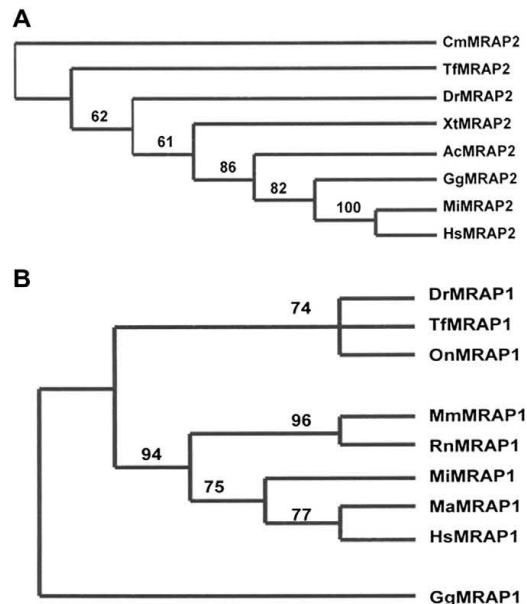


V5-tagged MRAPs were measured by whole cell ELISA as previously reported [25]. Significance of differences between pairs was evaluated by Student's unpaired *t*-test and between groups by ANOVA with Tukey's test.

The functional expression of rainbow trout MC2R was also done in CHO cells. For these experiments 2  $\mu$ g of rainbow trout MC2R, MRAP and CRE-Luc reporter plasmid were transfected using a Cell Line Nucleofector Kit (Amaya Inc.) with solution T and program U-23, and seeded on 96-well plate at a density of  $10^5$  cells/well. 48 h after transfection, cells were stimulated with appropriate ligands in serum-free CHO media for 4 h at 37 °C. At the end of the incubation, 100  $\mu$ l of Bright-Glo luciferase assay reagent (Promega Inc.) were applied to each well and incubated for 5 min at room temperature. Luminescence was measured with Bio-Tek Synergy HT plate reader. All experimental treatments were performed in triplicates, and statistical differences were determined using unpaired Student's *t*-test for equal variance with Kaleidagraph software. Significance was set at  $P \leq 0.05$ .

### 2.5. Phylogenetic analysis of MRAP sequences

The amino acid sequences of MRAP1 and MRAP2 were manually aligned [8]. The web site <http://www.ensembl.org/Multi/blastview> was used to search for MRAP-related sequences in the following species: Human – *Homo sapiens*, Monkey – *Macaca mulatta*, Lemur – *Microcebus murinus*, Rat – *Rattus norvegicus*, Mouse – *Mus musculus*; Chicken – *Gallus gallus*; Lizard – *Anolis carolinensis*, Frog – *Xenopus (Silurana) tropicalis*, bony fishes – *Danio rerio* and *Takifugu rubripes*. The *Oreochromis niloticus* MRAP1 (ENSUSP00000108774) was reported by Algulleiro et al. [1]. The web site <http://esharkgenome.imcbs-star.edu.sg/>, was used to search for MRAP-related sequences in the genome of the cartilaginous fish, *Callorhynchus milii* [29].



**Fig. 2.** Maximum parsimony analysis of MRAP1 and MRAP2. Maximum parsimony analysis was done using the PAUP 4.0 program (Sinauer). The data set was analyzed using the "exhaustive" algorithm, and the bootstrap values, based on 1000 replicates, are shown. The trees are unrooted. (A) Analysis of MRAP2 sequences. (B) Analysis of MRAP1 sequences.

Maximum Parsimony Analysis was done using the PAUP 4.0 program (Sinauer). The data set was analyzed using the exhaustive search mode algorithm, and the bootstrap values, based on 1000 replicates, are shown in Fig. 2.

## 3. Results

### 3.1. Phylogenetic analysis of vertebrate MRAPs

Fig. 1 presents the alignment of a representative set of MRAP1 and MRAP2 amino acid sequences. In order to align the two sets of paralogs, 23 gaps were required. This is an exceptionally large number for two paralogs gene products. The alignment revealed: (a) sites with high primary sequence identity (at least 70%) in both paralogs (gray), (b) sites with high primary sequence identity (at least 66%) in MRAP1 orthologs only (yellow), (c) sites with high primary sequence identity (at least 80%) in mammalian MRAP1 orthologs only (pink); and (d) sites with high primary sequence identity (at least 75%) in MRAP2 orthologs only (green). Although the overall sequence identity of the two paralogs was only 14%, it should be noted that both paralogs have: an N-linked glycosylation site near the N-terminal (position 9; Fig. 1), the amino acid motif Y/WEYY in the domain requiring for trafficking MC2R to the plasma membrane, a remarkable degree of sequence identity in the domain required for forming an ortholog specific homodimer (positions 43–54), and 46% of the positions in the transmembrane spanning domain were highly conserved. By contrast, the C-terminal region of the MRAP1 orthologs is highly divergent, and a construct of mammalian MRAP1 lacking the C-terminal domain still was found to be functional [12]. In the C-terminal of the MRAP2 orthologs there are several conserved amino acid motifs (positions shaded in green; Fig. 1). Although the functional significance of these amino acid motifs is unknown, these conserved regions were useful for the alignment of MRAP2 sequences.

Maximum parsimony analysis of the MRAP2 sequences yielded a single most parsimonious tree with a set of branching clades that corresponded to the phylogeny of the vertebrates (Fig. 2A). The bootstrap values for the amniote tetrapod clades were robust. However, the maximum parsimony analysis of the MRAP1 sequences was more complex. This analysis yielded three equally parsimonious trees, and the consensus tree is presented in Fig. 2B. The sequences split into a mammalian clade, a bony fish clade, and the chicken MRAP1 sequence did not group with either clade. The bootstrap values for the mammalian clade were strong, and this clade could be further divided into a rodent clade (mouse and rat MRAP1 orthologs) and a primate clade (lemur, monkey, and human MRAP1 orthologs). While the bony fish clade was clearly distinct from the mammalian clade, it was not possible to resolve the phylogenetic relationships of the three bony fish MRAP1 sequences using this algorithm. The position of the chicken MRAP1 sequence is problematic, and was probably influenced by the absence of other non-mammalian tetrapod MRAP1 sequences. Although the *Xenopus laevis* (<http://genome.jgi-psf.org/Xentr4/Xentr4.home.html>) and *Anolis carolinensis* ([http://www.ensembl.org/Anolis\\_carolinensis/](http://www.ensembl.org/Anolis_carolinensis/)) genome projects would appear to be likely sources to augment this analysis, a search of both genome data bases for MRAP1 has not revealed an ortholog for either species. Apart from this caveat, Fig. 2B would appear to indicate a tetrapod/mammalian trend and a bony fish trend in the evolution of MRAP1. From a functional standpoint these trends diverge at positions 30–33 in Fig. 1. The LDYL/I motif in mammalian MRAP1 is essential for facilitating the activation of mammalian MC2 receptors following an ACTH binding event [26]. Note that this motif is absent in all MRAP2 sequences (Fig. 1), and is altered in the teleost fish MRAP1 sequences. These observations would suggest that the

activation of a tetrapod MC2 receptor (e.g., frog MC2R) may be more robust in the presence of a tetrapod MRAP1, than in the presence of a fish MRAP1, and the converse may be true for the functional expression of a fish MC2 receptor (rainbow trout). The following experiments were done to test this hypothesis.

### 3.2. Functional activation of frog MC2 receptor in CHO cells

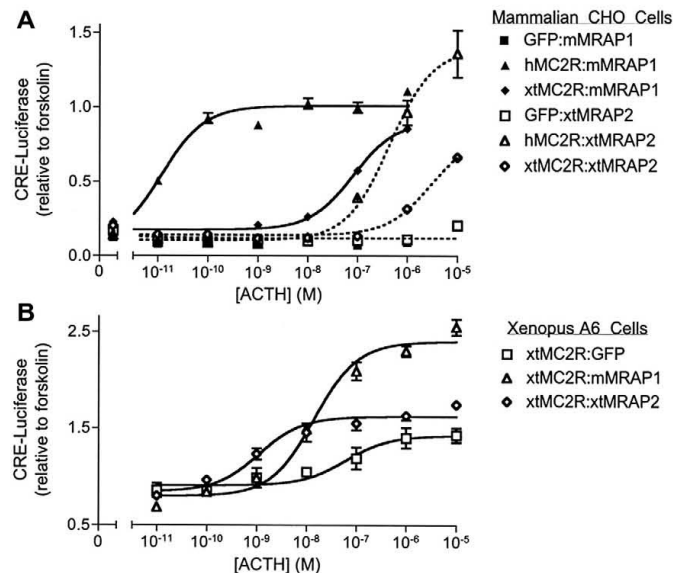
For this study the functional expression of frog MC2 receptors in CHO cells in the presence of a heterologous mouse MRAP1 (mMRAP1) was compared to the functional expression of the human receptor (hMC2R) in the presence of mMRAP1. For all experiments the CHO cells were transfected with a cAMP-responsive reporter construct, CRE-luciferase, and the MC2 receptor of interest was expressed either in the presence or absence of an MRAP construct. Cells were then challenged with different concentrations of human ACTH(1–24) [hACTH(1–24); Fig. 3A]. In agreement with published data [27], hMC2 receptors responded to subnanomolar concentrations of hACTH(1–24) with a robust cAMP response when hMC2 receptors were co-expressed with mMRAP1.

Since an MRAP1 ortholog has not been detected in the *X. tropicalis* genome but an MRAP2 ortholog is present, we tested the hypothesis that the frog receptor (xtMC2R) may only require the *Xenopus* MRAP2 (xtMRAP2) ortholog for functional expression. Co-transfection of xtMC2 receptors with xtMRAP2 generated a weak response when challenged with different concentrations of hACTH(1–24), and the hMC2 receptor behaved in a similar manner when co-transfected with xtMRAP2 (Fig. 3A). However, when xtMC2R was co-expressed in CHO cells with mMRAP1, a strong cAMP response was observed. It should be noted that almost  $10^5$ -fold higher concentrations of hACTH(1–24) were required to activate the xtMC2 receptor as compared to the activation of the mammalian MC2 receptor under similar conditions (Fig. 3A).

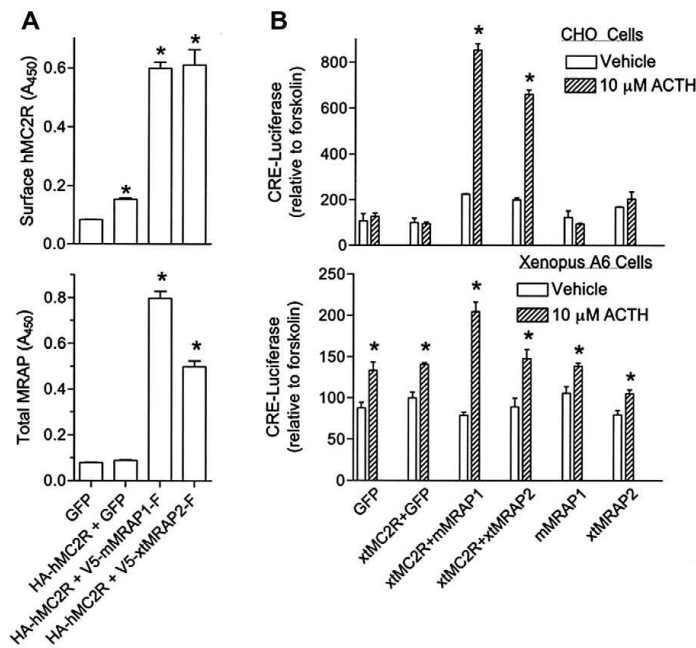
The weak response of the hMC2 receptor in the presence of xtMRAP2 could be the result of fewer hMC2 receptors trafficking to the plasma membrane in the presence of xtMRAP2 as compared to mMRAP1. However, as indicated by the cell surface ELISA (Fig. 4), both mMRAP1 and xtMRAP2 were expressed at equivalent levels on the surface of CHO cells, and both accessory proteins strongly increased the density of hMC2 receptors on the plasma membrane. We assume that xtMC2 receptors were also present on the plasma membrane in equivalent numbers in the presence of either mMRAP1 or xtMRAP2. As shown in Fig. 7D, xtMC2 receptors could be visualized on the surface of CHO cells when co-expressed with mMRAP1.

It is also conceivable that the lower sensitivity of xtMC2 receptors expressed in CHO cells for hACTH(1–24) is an indication that the mammalian cells might lack some unidentified chaperone required for effective signaling by the xtMC2 receptor. To test this hypothesis, we repeated the experiments presented in Fig. 3A in an established *Xenopus* cell line, the A6 kidney fibroblast line (Fig. 3B) [10]. A6 cells did not transfect well and responses were relatively small, but the results were quite similar to those obtained in the mammalian cells. The xtMC2 receptors responded better to hACTH(1–24) when they were expressed with mMRAP1 than with xtMRAP2. Unlike CHO cells, the *Xenopus* A6 cells displayed a small cAMP response to hACTH(1–24) in the absence of a transfected receptor or MRAP, suggesting that hACTH(1–24) can activate some endogenous receptors. Responses of xtMC2 receptors in CHO and A6 cells to  $10 \mu\text{M}$  ACTH, presumably a maximally effective concentration, are summarized in Fig. 4B.

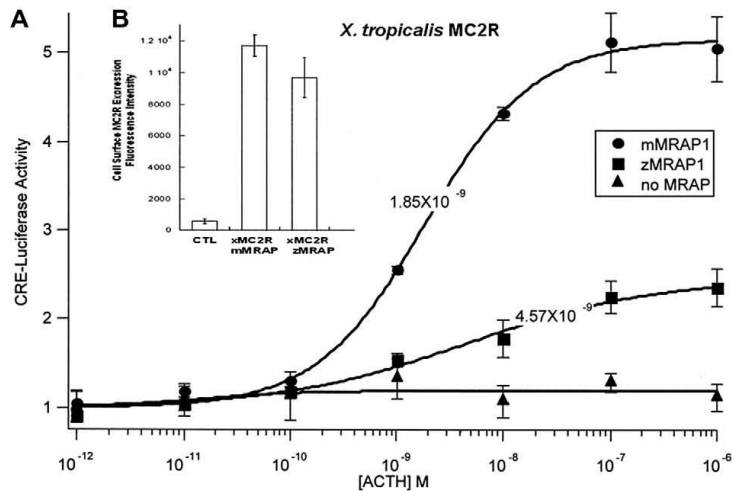
Finally, when the xtMC2 receptor was co-expressed with a fish MRAP1 (zebrafish MRAP1; zbMRAP1), there was a decline in the absolute amount of cAMP produced by CHO cells expressing xtMC2 receptors and zbMRAP1, as compared to CHO cells expressing xtMC2 receptor and mMRAP1 (Fig. 5A). In addition, xtMC2



**Fig. 3.** Functional expression of frog MC2R in CHO cells. Either CHO cells (A) or *Xenopus* A6 kidney fibroblasts (B) were transfected with cDNA encoding CRE-luciferase with or without hMC2 receptor, xtMC2 receptor, mMRAP1 and/or xtMRAP2. The next day cells were incubated for 5 h with different concentrations of ACTH before measurement of luciferase activity. For expression of MRAP and hMC2R in CHO cells, the  $EC_{50}$  values for ACTH were  $1.17 \times 10^{-11}$  M and  $4.45 \times 10^{-7}$  M with mMRAP1 and xtMRAP2, respectively. In CHO cells expressing xtMC2 receptor, the  $EC_{50}$  was  $8.09 \times 10^{-8}$  M with mMRAP1 but could not be established with no MRAP or xtMRAP2. (B) In *Xenopus* A6 cells,  $EC_{50}$  values were  $7.06 \pm 1.11 \times 10^{-8}$ ,  $1.31 \pm 0.12 \times 10^{-8}$  and  $0.43 \pm 0.22 \times 10^{-9}$  M ACTH for xtMC2 receptor expressed with no accessory protein, mMRAP1 and xtMRAP2, respectively.



**Fig. 4.** ELISA measurements of MRAP and hMC2R on CHO cells. (A) MRAP and hMC2 receptor expression. CHO cells were transfected as shown. The levels of HA-hMC2 receptor on the cell surface and total V5-tagged MRAPs were measured by ELISA. \* $P < 0.01$  vs. control with GFP alone. (B) Maximal responses of xMC2 receptors expressed in CHO or *Xenopus* A6 cells. Cells were transfected as shown, grown overnight, and incubated for 5 h with  $10^{-8}$  M ACTH before measurement of luciferase activity. \* $P < 0.01$  vs. no ACTH.



**Fig. 5.** Functional expression of frog MC2R with mMRAP1 or zMRAP1. (A) To determine how the xMC2R would interact with zebrafish (zf) MRAP1, equal numbers of CHO cells were either co-transfected with the CRE-luciferase cDNA, xMC2R cDNA, and zMRAP1 cDNA or co-transfected with CRE-luciferase cDNA, xMC2R cDNA and mMRAP1 cDNA. After 48 h in culture, the cells were incubated with different concentrations of hACTH(1–24) and luciferase activity was measured after 4 h of stimulation. The EC<sub>50</sub> value for xMC2R/zMRAP1 was nearly 2.5-fold greater than xMC2R/mMRAP1, and the maximum response for xMC2R/zMRAP1 was lower than the response in cells expressing xMC2R/mMRAP1. (B) The levels of V5-tagged xMC2R receptor on the cell surface in the presence of either mMRAP1 or zMRAP1 using an immunofluorescent cell surface assay procedure [30].



receptors showed a 2.5-fold drop in sensitivity to hACTH(1–24) when in the presence of zMRAP1 as compared to mMRAP1. The drop in rtMC2R stimulation by ACTH in cells co-expressing zebrafish MRAP1 was not due to fewer receptors on the plasma membrane as indicated by the cell surface ELISA analysis (Fig. 5B).

### 3.3. Functional activation of rainbow trout MC2 receptor in CHO cells

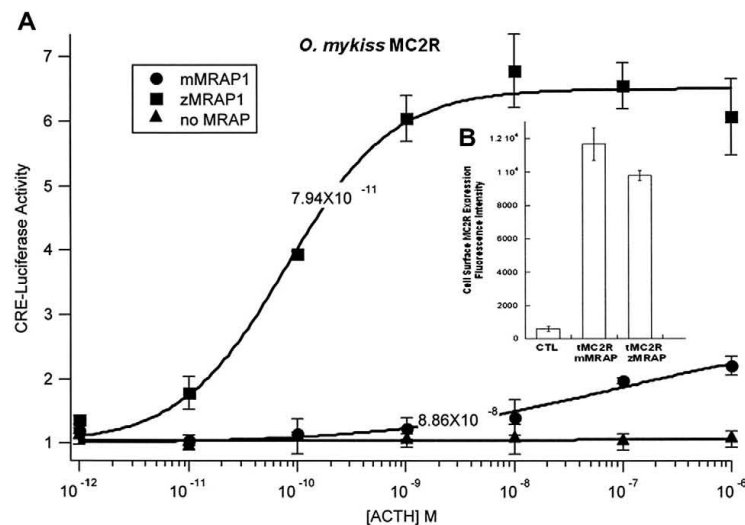
As expected, when a rainbow trout *mc2r* cDNA construct was transfected into CHO cells in the absence of an *mrp* construct, there was no functional expression of the receptor (Fig. 6A). An immunocytochemical analysis indicated that rainbow trout *mc2r* cDNA was expressed in CHO cells, but the immunopositive MC2 receptors were restricted to the cytosol, and no MC2 receptor immunoreactivity was apparent on the plasma membrane (Fig. 7A). However, when the rainbow trout *mc2r* cDNA construct was co-transfected with a mouse *mrp1* cDNA (Fig. 6A), the transfected cells produced cAMP in a dose dependent manner when stimulated with increasing concentrations of human ACTH(1–24). In agreement with this observation, it was possible to detect patches of rainbow trout MC2 receptor immunoreactivity on the plasma membrane of unpermeabilized transfected CHO cells (Fig. 7C). Note that the distribution of immunoreactive rtMC2R/mMRAP1 (yellow) was not uniform on the plasma membrane (Fig. 7C) as compared to the distribution of xtMC2R/mMRAP1 (Fig. 7D). However, mouse MRAP1 did facilitate the trafficking of the rainbow trout MC2 receptors to the plasma membrane, and the receptor could be activated by human ACTH(1–24). The  $EC_{50}$  ( $8.9 \times 10^{-8}$  M) for this dose response curve was relatively high.

This outcome may be a function of using a mammalian ACTH(1–24) analog to stimulate the cells instead of a teleost ACTH analog. However, a more plausible explanation may lie with the selection of the MRAP in the co-transfection experiment. When the rainbow trout *mc2r* cDNA construct was co-transfected with a zebrafish *mrp1* cDNA construct (Fig. 6A) both the  $EC_{50}$  ( $7.9 \times 10^{-11}$  M)

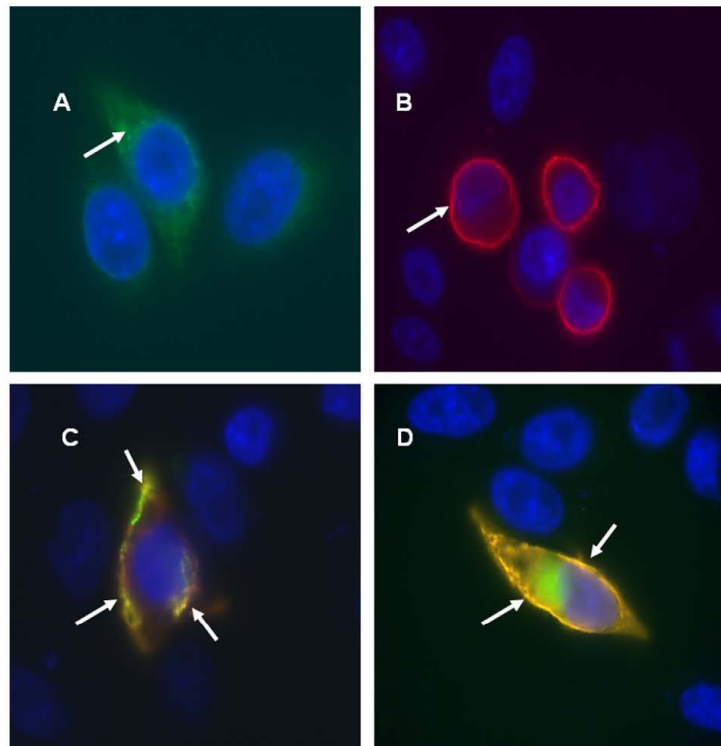
and ligand saturation levels were significantly higher in response to stimulation by hACTH(1–24) as compared to the co-transfection of CHO cells with rainbow trout MC2 receptor and mouse MRAP1. The dose response curve was in the same range as the co-expression of human MC2 receptor and mouse MRAP1 (Fig. 3). In addition, the rise in the stimulation of rtMC2R by ACTH in cells co-expressing zebrafish MRAP1 was not due to an increase in the number of rtMC2 receptors on the plasma membrane as indicated by the cell surface ELISA analysis (Fig. 6B).

## 4. Discussion

During the evolution of the MC2 receptor two properties have emerged that set this GPCR apart from the other members of the melanocortin (MC) receptor gene family: exclusive ligand selectivity for ACTH, and the requirement for interaction with MRAP1 in order to achieve full functional activation. The origin of these unique properties has not been resolved. While it is generally accepted that MC receptors are found only in the chordates, and that the ancestral MC receptor gene most likely emerged in an ancestral chordate lineage that predates the origin of the agnathan fishes, no MC receptor orthologs have been found in the genomes of extant protochordates [29]. However, two MC receptor orthologs have been characterized from the genome of an agnathan fish [11], but neither MC appears to be an ortholog of MC2R. Both lamprey MC receptors can be functionally expressed in HEK293 cells in the absence of MRAP, and both MC receptors can bind ACTH, as well as  $\alpha$ -MSH and be activated by these ligands [11]. Hence the origin of the *mc2r* gene must have occurred after the emergence of the ancestral gnathostomes and as a consequence of the genome duplication event that demarcates this stage in the evolution of the chordates [20]. Since unique ligand and MRAP requirements are features common to the MC2 receptor in at least two species of bony fishes [1,16], an amphibian (*X. tropicalis*), and in mammals



**Fig. 6.** Functional expression of rainbow trout MC2R in CHO cells. (A) To determine the activation parameters of rainbow trout (rt) MC2R in the presence of either zMRAP1 or mMRAP1, equal numbers of CHO cells were either co-transfected with the CRE-luciferase cDNA, rtMC2R cDNA, and zMRAP1 cDNA or co-transfected with CRE-luciferase cDNA, rtMC2R cDNA and mMRAP1 cDNA. After 48 h in culture, cells were incubated with different concentrations of hACTH(1–24) and luciferase activity was measured after 4 h of stimulation. The  $EC_{50}$  value for the rtMC2R/mMRAP1 was 300-fold greater than the rtMC2R/zMRAP1, and the hACTH(1–24) response was robust for the rtMC2R/zMRAP1. As a control rtMC2R was transfected into CHO cells in the absence of either MRAP1 cDNA. (B) The levels of V5-tagged rtMC2 receptor on the cell surface in the presence of either mMRAP1 or zMRAP1 using an immunofluorescent cell surface assay procedure [30].



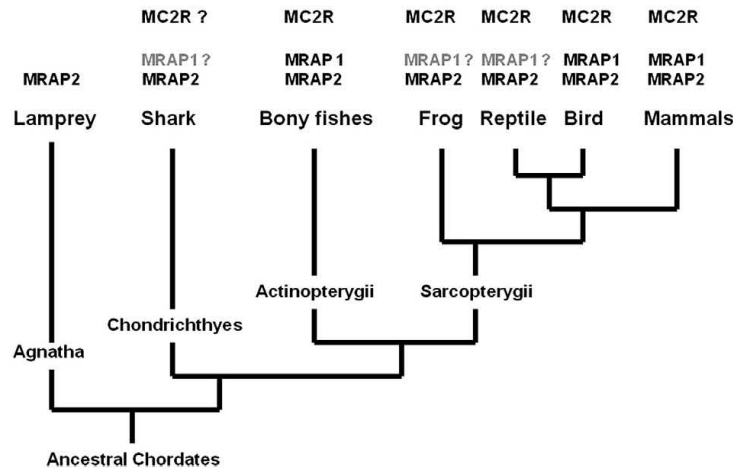
**Fig. 7.** Immunocytochemical analysis of CHO cells transfected with MC2R. (A) The rainbow trout MC2R construct with a V-5 Epitope tag was transfected into CHO cells in the absence of mMRAP1 and incubated in a chamber mounted onto a coated glass slide. The cells were permeabilized, fixed and immunostained as described in Section 2. The white arrow indicated immunopositive rtMC2R was restricted to the cytosol and does not appear on the cell surface. (B) The mouse MRAP1 construct with a FLAG epitope tag was transfected alone into CHO cells and after 48 h, the unpermeabilized cells were fixed and immunostained as described in Section 2. Nuclei were stained with DAPI. Note the intense staining of mMRAP1 on the plasma membrane (arrow) and that that trafficking of MRAP1 can occur when MC2R is not present. (C) The rtMC2R/V-5 construct and the mMRAP/FLAG construct were co-transfected into CHO cells and incubated as described above. The (unpermeabilized) cells were fixed and immunostained with the V-5 and FLAG antisera. Note the yellow reaction (white arrows) on the plasma membrane indicative of the co-localization of rtMC2R and mMRAP on the plasma membrane. (D) The *X. tropicalis* xtMC2R/V-5 construct and the mMRAP/FLAG construct were co-transfected into CHO cells and incubated as described above. The (unpermeabilized) cells were fixed and immunostained with the V-5 and FLAG antisera. Note the yellow reaction (white arrows) on the plasma membrane indicative of the co-localization of rtMC2R and mMRAP on the plasma membrane (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

[6], these traits must have been acquired prior to the divergence of the ancestral Actinopterygii (ray finned fishes) and the ancestral Sarcopterygii (lobed finned fishes and tetrapods) over 400 million years ago. An understanding of the transitions that occurred in a proto-MC2R ortholog may still be apparent from studies on the MC receptors in extant cartilaginous fishes [29] or perhaps in the older lineages of ray-finned fishes, such as the extant chondrosteans and holosteans, or in an extant lineage of the lobe-finned fishes, such as the lungfishes [19]. At present, then, it would be reasonable to propose that some, as yet, undetermined feature in the tertiary structure of bony vertebrate MC2 receptors prevents  $\alpha$ -MSH and the other MSH ligands from binding and activating the MC2 receptor but accommodates ACTH for this purpose. In addition, and coincidentally, an as yet undetermined feature in the tertiary structure of bony vertebrate MC2 receptors allows for a positive interaction between the MC2 receptor and MRAP1 resulting in activation following an ACTH binding event. This study has focused on this MRAP1/MC2R interaction.

The presence of a MRAP2 ortholog in the lamprey genome [29] would indicate that the *mrp* gene appeared relatively early in the evolution of the vertebrates, but prior to the second genome duplication event that occurred with the emergence of the gnathostomes [20].

Given this time line, the presence of *mrp1* and *mrp2* paralogs in representatives of the bony fishes, an avian species (*Gallus gallus*) [29] and in several mammalian genomes is expected (Fig. 8). What was not expected is the apparent absence of an *mrp1* ortholog in the genomes of the cartilaginous fish, *Callorhynchus milii*, the amphibian *Xenopus tropicalis* [29], and the reptile *Anolis carolinensis*. Presumably in all three of these lineages an *mrp1* orthologous gene has been secondarily lost. As indicated in this study, the assumption of a secondary loss of the *mrp1* ortholog in some gnathostome lineages would appear to be, from a functional standpoint, an unsatisfactory explanation as will be discussed presently.

What does emerge from Figs. 1 and 8 is that following the duplication of the ancestral *mrp* gene, the MRAP1 and MRAP2 paralogs have retained high primary sequence identity in the N-terminal domains required for trafficking MC2R to the plasma membrane (positions 26–29; Fig. 1), in the homodimer domain (43–50; Fig. 1), and in the transmembrane spanning domain (50–68; Fig. 1). Hence as has already been shown for mammals [12,31] and for the zebrafish [1], the trafficking of MC2R from the ER to the plasma membrane can be achieved through an interaction with either MRAP1 or MRAP2, and does not occur if either MRAP paralog



**Fig. 8.** Phylogenetic distribution of MRAP1 and MRAP2 in the major groups of vertebrates. The sequence of MRAP2 has been detected in the genome of *Petromyzon marinus* (lamprey) [29], *Callorhynchus milii* (shark) [29], several bony fishes [1,12,31], *Xenopus tropicalis* (Frog; this study), *Anolis carolinensis* (Reptile; [http://www.ensembl.org/Anolis\\_carolinensis/](http://www.ensembl.org/Anolis_carolinensis/)), *Gallus gallus* (Bird) [12,31], and several mammals (Mammals) [31]. The sequence of MRAP1 has been detected in the genome of *Danio rerio* (Bony fish) [1], *Gallus gallus* (Bird) [12,31], and several mammals [17,31].

was not present. Therefore, there must be a domain on mammalian and zebrafish MC2Rs that docks with the MRAP paralog in the ER, and as a result allows for the trafficking of MC2R out of the ER. Furthermore for full functional activation, the MC2R must be in contact with MRAP1 at the plasma membrane [1,12,31], and this feature appears to reside in the amino acid motif at positions 30–33 (Fig. 1) that is present in the MRAP1 orthologs, but is absent in the MRAP2 paralogs.

Given these observations, the apparent absence of an MRAP1 ortholog from the *X. tropicalis* genome may indicate that: (a) the *X. tropicalis* (xt) MC2 receptor does not require MRAP for trafficking to the plasma membrane and for activation, or (b) *X. tropicalis* MRAP2 facilitates these functions. However, as revealed in Fig. 3A, expression of xtMC2R in CHO cells in the absence of an MRAP results in no activation by hACTH(1–24) because trafficking of xtMC2R to the plasma membrane has been blocked (data not shown). In this regard, the *X. tropicalis* MC2 receptor is behaving in the same manner as a bony fish or mammalian MC2 receptor. In addition when xtMC2R was co-expressed with *X. tropicalis* MRAP2, the receptor could only be marginally activated at the highest dose of hACTH(1–24). In this experiment it appears that *X. tropicalis* MRAP2 facilitated the trafficking of xtMC2R to the plasma membrane, but because *X. tropicalis* MRAP2 lacks the LDYL/I motif this MRAP could not enhance the interaction between xtMC2R and hACTH(1–24). However, when xtMC2R was co-expressed with mouse MRAP1, trafficking of the receptor to the plasma membrane was effectively achieved (Fig. 7D), and xtMC2R responded to hACTH(1–24) in a dose dependent manner (Fig. 3). Furthermore, the *X. tropicalis* MC2 receptor could only be activated by hACTH(1–24) and not by NDP $\alpha$ -MSH at doses as high as  $10^{-6}$  M when co-expressed with mouse MRAP1 (data not shown). Clearly the *X. tropicalis* MC2 receptor has the predicted domain required for interacting with mouse MRAP1. Hence, the combination of xtMC2R/mMRAP1 can be used in heterologous expression studies in mammalian cells to analyze the interactions that occur between ACTH and the *X. tropicalis* MC2 receptor during an activation event. These observations however raise the question of how does the *X. tropicalis* MC2 receptor function in *X. tropicalis* interrenal cells if an MRAP1 ortholog is not in the frog genome. Assuming that the

*X. tropicalis* genome project is complete, some other accessory protein may have taken over the function of MRAP1. Other GPCR accessory proteins such as the RAMPs and RTPs have been identified [3,13] and none of these chaperones appear to share a common origin. Thus the intercession of a novel accessory protein is not unprecedented. The experiment with the frog A6 kidney cells was an attempt to address this question. The assumption was that since the frog A6 kidney cells are of mesodermal origin and have a common embryonic origin with the frog adrenocortical cells, perhaps the A6 cells express a similar subset of genes as the frog adrenocortical cells. However, as shown in Fig. 3B, expression of xtMC2R in A6 cells in the absence of an endogenous MRAP construct did not enhance the activation dose response curve for xtMC2R. Future studies should focus on the characterization of MRAP-related mRNAs from *X. tropicalis* adrenocortical cells.

When considering the results presented in Fig. 3A, was the activation of xtMC2R in the presence of mMRAP1 optimal? The human MC2 receptor was much more sensitive to stimulation by hACTH(1–24) in the presence of mMRAP1 as compared to the xtMC2R. This difference in sensitivity may reflect how well xtMC2R interacted with the mouse MRAP1. It is noteworthy that when xtMC2R was co-expressed with zebrafish MRAP1 in CHO cells (Fig. 5) there was a drop in both sensitivity for hACTH(1–24) and the amplitude of the dose response curve as compared to the xtMC2R/mMRAP1 co-transfection. This is another question that could be resolved from studies on frog adrenocortical cells.

The extremes in functional activation patterns were even greater for the heterologous expression of the rainbow trout MC2 receptor. As expected, the rainbow trout (rt) MC2 receptor could not be functionally expressed when transfected into CHO cells in the absence of an MRAP construct (Fig. 6). Immunocytochemical analysis of these cells indicated that the rtMC2R immunoreactivity was restricted to compartments in the cytosol and did not traffic to the plasma membrane (Fig. 7A). In the presence of mouse MRAP1, the rtMC2R could be activated by hACTH(1–24) in a dose dependent manner, and immunoactive rtMC2R could be detected on the plasma membrane of the transfected CHO cells (Fig. 7C). However, the distribution of staining over the surface of the rtMC2R/mMRAP1 transfected cells (Fig. 7C) was more diffuse than the staining



pattern observed for the xtMC2R/mMRAP1 transfected cells (Fig. 7D). It would appear that the interaction between the rtMC2R and the mammalian MRAP1 is functional, but not optimal. In support of this conclusion, the co-transfection of rainbow trout MC2 receptor and the zebrafish MRAP1 in CHO cells yielded a robust dose response curve for hACTH(1–24) (Fig. 6).

Collectively, these experiments would suggest that the co-evolution of the MC2R gene and the MRAP1 gene has diverged to yield a bony fish trend with respect to the interaction between MC2R and MRAP1 and a tetrapod trend with respect to the interaction between MC2R and MRAP1. A corollary to this hypothesis is that there may be a further bifurcation of the tetrapod MC2R/MRAP1 trend between non-mammalian and mammalian tetrapods. Establishing the critical regions in bony fish and tetrapod MC2Rs that contribute to the ligand selectivity properties of these MC2Rs and their respective interactions with their lineage specific MRAP1 can be effectively explored through the heterologous expression in mammalian cells. For each MC2R studied using expression in heterologous mammalian cells, it will be important to analyze ACTH sensitivity by the adrenal/interrenal cells of that species. The possible presence of novel MCR accessory proteins, and the potential interaction between MC2R and MC5R in some adrenal/interrenal cells, as documented for the barfin flounder head kidney [16], add to the complex interactions involved in the regulation of the hypothalamus/pituitary/adrenal/interrenal axis.

Finally, it should be noted that well characterized, heterologous expression systems such as CHO cells are widely used to study receptor signaling. Advantages include relatively easy transfection procedures, high levels of expression of receptor, accessory proteins and reporters, and strong cAMP-dependent responses. The ACTH responses detected in CHO cells in this study were entirely dependent on the transfected receptors introduced into the CHO cells. Homologous cell lines are not available for several of the species of receptors and accessory proteins tested in this study. Hence these studies using CHO cells have provided insights into how xtMC2R and rtMC2R could potentially perform when endogenously expressed in interrenal cells, and are useful for designing experiments to evaluate endogenous expression in frog and fish interrenal cells, respectively.

## Acknowledgments

This research was supported by NSF Grant IOB 0516958 (R.M.D.) and NIH Grant DK19974 (P.M.H.).

## References

- [1] M.J. Agulleiro, S. Roy, E. Sanchez, S. Puchol, N. Gallo-Payet, J.M. Cerda-Reverter, Role of melanocortin receptor accessory proteins in the function of zebrafish melanocortin receptor type 2, *Mol. Cell. Endocrinol.* 320 (2010) 145–152.
- [2] M. Aluru, M.M. Vijayan, Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout, *Endocrinology* 149 (2008) 4577–4588.
- [3] M. Behrens, J. Bartelt, C. Reichling, M. Wining, C. Kuhn, W. Meyerhof, Members of RP and REEP gene families influence functional bitter taste receptor expression, *J. Biol. Chem.* 281 (2006) 20650–20659.
- [4] J.M. Cerda-Reverter, M.A. Agulleiro, R. Guillot, E. Sanchez, R. Ceinos, J. Rotlant, Fish melanocortin system. *Eur. J. Pharmacol.* doi:10.1016/j.ejphar.2010.10.108.
- [5] O.G. Chepur, G.G. Holz, A novel cyclic adenosine monophosphate responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein coupled receptor drug discovery efforts, *J. Biomol. Screen.* 12 (2007) 740–746.
- [6] R.D. Cone, Studies on the physiological functions of the melanocortin system, *Endocr. Rev.* 27 (2006) 736–749.
- [7] S.N. Cooray, T.-T. Chung, K. Mazhar, I. Szidonya, A.J.L. Clark, Bioluminescence resonance energy transfer reveals the adrenocorticotropin (ACTH)-induced conformational change of the activated ACTH receptor complex in living cells, *Endocrinology* 152 (2011) 495–502.
- [8] R.M. Does, D.A. Rubin, T.W. Quinn, Mini-review: is it possible to construct phylogenetic trees using polypeptide hormone sequences, *Gen. Comp. Endocrinol.* 103 (1996) 1–12.
- [9] F.L. Forti, M.H.S. Dias, H.A. Armelin, H.A. ACTH, Receptor: ectopic expression, activity and signaling, *Mol. Cell. Biochem.* 293 (2006) 147–160.
- [10] R.J. Gantress, N. Cohen, G.D. Maniero, *Xenopus* as an experimental model for studying evolution of hsp-immune system interactions, *Methods* 32 (2004) 42–53.
- [11] T. Haitina, J. Klovins, A. Takahashi, M. Lowgren, A. Ringholm, J. Enberg, H. Kawachi, E.T. Larson, R. Fredriksson, H.B. Schioth, Functional characterization of two melanocortin (MC) receptors in lamprey showing orthology to the MC1 and MC4 receptor subtypes, *BMC Evol. Biol.* 7 (2007) 101–115.
- [12] P.M. Hinkle, J.A. Sebag, Structure and function of the melanocortin 2 receptor accessory protein, *Mol. Cell. Endocrinol.* 300 (2009) 25–31.
- [13] D.L. Hay, D.R. Poyner, P.M. Sexton, GPCR modulation by RAMPs, *Pharmacol. Ther.* 109 (2006) 173–197.
- [14] J. Klovins, T. Haitina, D. Fridmanis, Z. Kilanova, I. Kapa, R. Fredriksson, N. Gallo-Payet, H.B. Schioth, The melanocortin system in *Fugu*: determination of POMC/AGRP/MCR gene repertoire and synteny, as well as pharmacology and anatomical distribution of the MCRs, *Mol. Biol. Evol.* 21 (2004) 563–579.
- [15] J. Klovins, T. Haitina, A. Ringholm, M. Lowgren, D. Fridmanis, M. Slaidina, et al., Cloning of two melanocortin (MC) receptors in spiny dogfish, *Eur. J. Biochem.* 271 (2004) 4320–4331.
- [16] Y. Kobayashi, H. Chiba, T. Yamanome, H.B. Schioth, A. Takahashi, Melanocortin receptor subtypes in interrenal cells and corticotropic activity of  $\alpha$ -melanocyte-stimulating hormones in barfin flounder, *Verasper moseri*, *Gen. Comp. Endocrinol.* 170 (2011) 558–568.
- [17] L.A. Metherell, J.P. Chapple, S. Cooray, A. David, C. Becker, F. Ruschendorf, D. Naville, M. Begeot, B. Khoo, P. Nurnberg, A. Huebner, M.E. Cheetham, A.J. Clark, Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2, *Nat. Genet.* 37 (2005) 166–170.
- [18] K.G. Mountjoy, L.S. Robbins, M.T. Mortrud, R.D. Cone, The cloning of a family of genes that encode the melanocortin receptors, *Science* 257 (1992) 1248–1251.
- [19] J.S. Nelson, *Fishes of the World*, third ed., John Wiley and Sons, Inc., New York.
- [20] S. Ohno, U. Wolf, N.B. Atkins, Evolution from fish to mammals by gene duplication, *Hereditas* 59 (1968) 169–187.
- [21] M. Rachel, H. El Mourabit, A. Buronfosse, A. Blondet, D. Naville, M. Begeot, A. Penhoat, Expression of the human melanocortin-2 receptor in different eukaryotic cells, *Peptides* 26 (2005) 1842–1847.
- [22] A. Ringholm, J. Klovins, R. Fredriksson, N. Poliakova, E.T. Larson, J.P. Kukkonen, D. Larhammar, H.B. Schioth, Presence of melanocortin (MC4) receptor in spiny dogfish suggests an ancient vertebrate origin of central melanocortin system, *Eur. J. Biochem.* 270 (2003) 213–221.
- [23] J. Robert, J. Gantress, N. Cohen, G.D. Maniero, *Xenopus* as an experimental model for studying evolution of hsp-immune system interactions, *Methods* 32 (2004) 42–53.
- [24] S. Roy, M. Rachel, N. Gallo-Payet, Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms  $\alpha$  and  $\beta$  in isogenic human embryonic kidney 293 cells, *Mol. Endocrinol.* 21 (2007) 1656–1669.
- [25] J.A. Sebag, P.M. Hinkle, Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers, *Proc. Natl. Acad. Sci. USA* 104 (2007) 20244–20249.
- [26] J.A. Sebag, P.M. Hinkle, Regions of melanocortin w (MC2) receptor accessory protein necessary for dual topology and MC2 receptor trafficking and signaling, *J. Biol. Chem.* 284 (2009) 610–618.
- [27] J.A. Sebag, P.M. Hinkle, Regulation of G-protein signaling: specific dominant-negative effects of melanocortin 2 receptor accessory protein 2, *Sci. Signal.* 3 (2010) 116 ra28.
- [28] C.M. Tan, A.E. Brady, H.H. Nickols, Q. Wang, L.E. Limbird, Membrane trafficking of G-protein-coupled receptors, *Annu. Rev. Pharmacol. Toxicol.* 44 (2003) 559–609.
- [29] A. Vastermark, H.B. Schioth, The early origin of melanocortin receptors, agouti-related peptides, agouti signaling peptide, and melanocortin receptor accessory proteins with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus, *Eur. J. Pharm.* doi:10.1016/j.ejphar.2010.10.106.
- [30] T.R. Webb, L.F. Chan, S.N. Cooray, M.E. Cheetham, J.P. Chapple, A.J. Clark, Distinct melanocortin 2 receptor accessory protein domains are required for melanocortin 2 receptor interaction and promotion of receptor trafficking, *Endocrinology* 150 (2009) 720–726.
- [31] T.R. Webb, J.L. Clark, Minireview: The melanocortin 2 receptor accessory proteins, *Mol. Endocrinol.* 24 (2010) 475–484.

## **APPENDIX 3**

## Identification of an MRAP-Independent Melanocortin-2 Receptor: Functional Expression of the Cartilaginous Fish, *Callorhynchus milii*, Melanocortin-2 Receptor in CHO Cells

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Phylogenetic analyses indicate that the genome of the cartilaginous fish, *Callorhynchus milii* (elephant shark), encodes a melanocortin-2 receptor (MC2R) ortholog. Expression of the *elephant shark mc2r* cDNA in Chinese hamster ovary (CHO) cells revealed that trafficking to the plasma membrane and functional activation of the receptor do not require coexpression with an exogenous *melanocortin receptor-2 accessory protein (mrp)* cDNA. Ligand selectivity studies indicated that elephant shark MC2R-transfected CHO cells produced cAMP in a dose-dependent manner when stimulated with either human ACTH (1–24) or [Nle<sup>4</sup>, D-Phe<sup>7</sup>]-MSH. Furthermore, the order of ligand selectivity when elephant shark MC2R-transfected CHO cells were stimulated with cartilaginous fish melanocortins was as follows: ACTH (1–25) =  $\gamma$ -MSH =  $\delta$ -MSH >  $\alpha$ -MSH =  $\beta$ -MSH. Elephant shark MC2R is the first vertebrate MC2R ortholog to be analyzed that does not require melanocortin receptor-2 accessory protein 1 for functional activation. In addition, elephant MC2R is currently the only MC2R ortholog that can be activated by either ACTH- or MSH-sized ligands. Hence, it would appear that MC2R dependence on melanocortin receptor-2 accessory protein 1 for functional activation and the exclusive selectivity of this melanocortin receptor for ACTH are features that emerged after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes more than 400 million years ago. (*Endocrinology* 153: 0000–0000, 2012)

The salient features that distinguish the mammalian melanocortin-2 receptor (MC2R) from other mammalian melanocortin receptors (*i.e.* MC1R, MC3R, MC4R, and MC5R) are the following: 1) the requirement for interaction with melanocortin receptor-2 accessory protein 1 (MRAP1) to facilitate both the trafficking of MC2R to the plasma membrane and the functional activation of MC2R at the plasma membrane (1, 2), and 2) the strict ligand selectivity of MC2R for ACTH coupled with the inability of MSH-sized ligands (*i.e.*  $\alpha$ -MSH,  $\beta$ -MSH, and  $\gamma$ -MSH) to activate this melanocortin receptor (MCR) (3). As a result, MC2R is the most functionally constrained member of the MCR family in terms of ligand selectivity. These unique features have been observed in several studies on mammalian MC2R (4, 5), and relatively recently these same constraints on activation were observed for the MC2R of the teleosts, *Danio rerio* (zebrafish) (6) and *Onchocorhynchus mykiss* (rainbow trout) (7), and the MC2R of

the amphibian, *Xenopus tropicalis* (8). Based on these observations, it would be reasonable to propose that the MC2R of all the bony vertebrates (*i.e.* the ray finned fishes, lobe finned fishes, and tetrapods) have these features in common.

The presence of at least five *mcr* paralogous genes in the genomes of teleosts and tetrapods appears to have been the result of two genome duplication events and at least one local gene duplication event that have occurred during the radiation of the chordates (9). In this scenario, duplications of an ancestral *mcr* gene, of presumably protochordate origin, would yield five *mcr* paralogs in the ancestral jawed vertebrates (gnathostomes) (10). During the subsequent radiation of the gnathostomes (*i.e.* cartilaginous fishes, bony fishes, and tetrapods) the evolution of the MCR gene family has been influenced by gene loss (11) and lineage-specific gene duplication events (12). In this regard, gene loss may be most evident in the cartilaginous

ISSN Print 0013-7227 ISSN Online 1945-7170

Printed in U.S.A.

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doi: 10.1210/en.2012-1482 Received April 29, 2012. Accepted August 1, 2012.

Abbreviations: CHO, Chinese hamster ovary; DAPI, 4',6'-diamino-2-phenylindole; EC2, extracellular loop 2; MCR, melanocortin receptor; MC2R, melanocortin-2 receptor; mMRAP1, mouse *mrp1*; MRAP1, MC2R accessory protein 1; NDP, [Nle<sup>4</sup>, D-Phe<sup>7</sup>]; TM, transmembrane domain; zfMRAP1, zebrafish *mrp1*.

fishes, one of the stem ancestral gnathostome groups (13). Studies on the shark, *Squalus acanthias* (14, 15), revealed the presence of orthologs of MC3R, MC4R, and MC5R in the *S. acanthias* genome. However, a recent analysis of the genome project for the holocephalan cartilaginous fish, *Callorhynchus milii* (elephant shark) uncovered orthologs only for MC1R, MC2R, and MC3R (10). The identity of the elephant shark MCRs was based on a maximum likelihood analysis, which placed each elephant shark sequence in a clade with the corresponding human MCR ortholog (10). In this regard, the detection of a putative elephant shark MC2R was of particular interest since previous studies on two species of elasmobranch sharks had not detected an MC2R ortholog in the genomes of these cartilaginous fishes (14, 15, 16). Maximum parsimony analysis of elephant shark MC2R, human MCRs, and zebrafish MCRs (Fig. 1; Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>) also placed the elephant shark MC2R in the same clade with human and zebrafish MC2R.

A comparison of several gnathostome MC2R sequences (Supplemental Fig. 2) indicates that elephant shark MC2R has several features in common with the gnathostome MC2R including a short N-terminal domain with two N-linked glycosylation sites, a short C-terminal domain, and at least 50% primary sequence identity in transmembrane regions 6 and 7, intracellular loops 1 and 2, and extracellular loop 3. However, these sequence comparisons do not reveal whether elephant shark MC2R has

the same pharmacological properties as teleost or tetrapod MC2 receptors. Working on the assumption that all vertebrate MC2R orthologs are MRAP1 dependent and can be activated only by ACTH but not by  $\alpha$ -MSH, a V-5 epitope-tagged *elephant shark mc2r* cDNA was transiently transfected into a heterologous mammalian cell expression system [Chinese hamster ovary (CHO) cells] in the presence and absence of either mammalian or teleost MRAP1 orthologs or an elephant shark MRAP2 ortholog, and ligand selectivity analyses were done with either mammalian melanocortins or cartilaginous fish melanocortins. These experiments revealed that the elephant shark MC2R is an MRAP-independent MCR that can be activated by either ACTH or MSH-sized ligands with varying degrees of efficacy.

## Materials and Methods

### MC2R and MRAP constructs

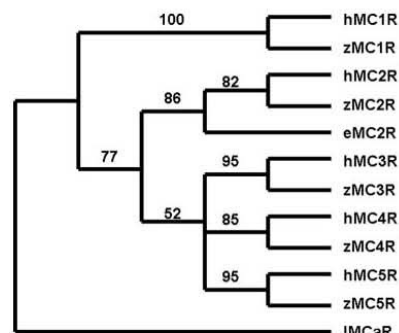
Elephant shark (*C. milii*) MC2R (accession no. FAA704.1) and human MC2R (accession no. AA067714.1), were synthesized by GenScript (Piscataway, NJ) with an N-terminal V-5 epitope tag and inserted into a pcDNA3.1+ vector. Mouse (*Mus musculus*) MRAP1 (accession no. NM\_029844), zebrafish (*Danio rerio*) MRAP1 (accession no. XR\_117835), and elephant shark (*C. milii*) MRAP2, (accession no. BR000861) were individually synthesized by GenScript with an N-terminal FLAG epitope tag and were separately inserted into pcDNA3.1+ vectors.

### Tissue culture

The experiments were done in transiently transfected CHO cells (American Type Culture Collection, Manassas, VA). The CHO cells were grown at 37°C in a humidified 5% CO<sub>2</sub> incubator in Kaighn's modification of Ham's F-12 (2 mM glutamine, 1500 mg/liter bicarbonate) with 10% fetal bovine serum, 5 ml penicillin/streptomycin, and 1 ml normocin.

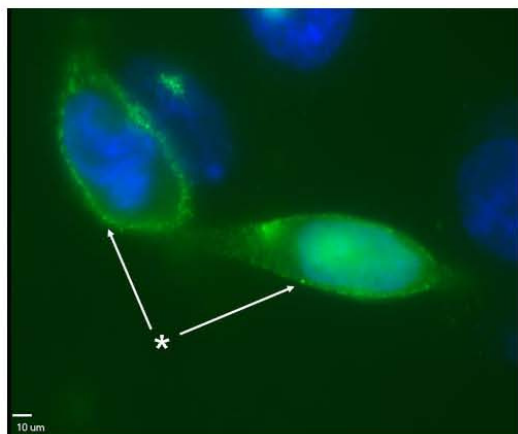
### Immunofluorescence microscopy

CHO cells were grown in two-well chamber slides ( $1 \times 10^5$  cells/well) for 24 h before transfection. The cells were transfected with 1  $\mu$ g of *elephant shark mc2r* cDNA construct using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and Opti-MEM (Cellgro, Manassas, VA). After 24 h in culture, the cells were fixed with 4% paraformaldehyde for 15 min, and the immunofluorescence analysis was done on permeabilized and nonpermeabilized cells (Fig. 2) as described previously (8). The primary antibody, a mouse monoclonal anti-V5 antibody, was diluted 1:100 in PBS + 1% BSA solution. The cells were incubated with the primary antibody for 1 h at 37°C. After a wash step, the cells were incubated with the secondary antibody, donkey anti-mouse antibody linked to Alexa-Fluor488 (1:400 in PBS + 1% BSA) for 45 min at 37°C to visualize MC2R immunofluorescence (Fig. 2, green). Coverslips were mounted onto slides using Vectashield (Vector Laboratories, Burlingame, CA), and nuclei were stained with 4',6'-diamino-2-phenylindole (DAPI) (Fig. 2, blue). The



**FIG. 1.** Maximum parsimony analysis of human, zebrafish, and elephant shark MCR. Maximum parsimony analysis was done using the branch and bound analysis algorithm (PAUP 4.3). The following sequences were aligned as described by Dores et al. (35): human, MC1R (AAD41355.1), MC2R (AA067714.1), MC3R (AA072726.1), MC4R (AA092061.1), and MC5R (NP\_005904.1); zebrafish, MC1R (AA162848.1), MC2R (NP\_775386.1), MC3R (AA024744.1), MC4R (AAL85494.1), and MC5R (NP\_775386.1); and *C. milii* MC2R (FAA704.1). The sequence of *Lampetra fluviatilis* MCRa receptor (IMCaR; ABB36647.1) was used as the outgroup. The bootstrap values in this figure were derived from 1000 replications.





**FIG. 2.** Immunofluorescence detection of elephant shark MC2R expressed in CHO cells. In CHO cells the V5-tagged elephant shark MC2R was detected on the surface of nonpermeabilized CHO cells (white arrows; marked by the asterisk). Green, Elephant shark MC2R (Alexa-Fluor488); blue, nuclei (DAPI). Space bar, 10  $\mu$ m.

control for this experiment was the absence of immunofluorescence in permeabilized transfected cells following incubation with the V5-specific secondary antiserum only (Fig. 3A).

To demonstrate that the clone of CHO cells used in this study lacks endogenous MRAP, cells were transfected with a human MC2R cDNA (1  $\mu$ g) as described above, and immunofluorescence was done on nonpermeabilized cells (Fig. 3B) and permeabilized cells (Fig. 3C). Finally, to demonstrate that exogenous MRAP can be functionally expressed in the clone of CHO cells used in this study, cells were cotransfected with a human MC2R cDNA (1  $\mu$ g) and a mouse MRAP1 cDNA (1  $\mu$ g) as described previously (8), and immunofluorescence was done on nonpermeabilized cells (Fig. 3, D–F). Images were obtained using a  $\times 100$  oil immersion objective (Zeiss Plan-NEOFUAR; Carl Zeiss, New York, NY) with a fluorescence microscope equipped with a Hamamatsu digital camera (Hamamatsu, Bridgewater, NJ) and a Sutter excitation filter wheel with Semrock filters (Sutter Instruments, Navato, CA). All images were analyzed using SlideBook software (Intelligent Imaging Innovations, Inc, Denver, CO).

#### Functional expression assays

CHO cells were transfected with 2  $\mu$ g each of *elephant shark mc2r* cDNA and *cre-luc* reporter plasmid (17) either with an *mrp* cDNA construct or in the absence of an *mrp* cDNA construct using a cell line nucleofector kit (Amaxa, Inc.; www.lonza.com) with solution T and program U-023. The cells were then plated on a white, 96-well plate at a density of  $1 \times 10^5$  cells/well. After 48 h in culture, the transfected cells were stimulated with melanocortin ligands in serum-free CHO media for 4 h at 37°C at concentrations ranging from  $10^{-6}$  to  $10^{-12}$  M. After the incubation period, 100  $\mu$ l of Bright-Glo luciferase assay reagent (Promega Inc., Madison, WI) was applied to each well and incubated at room temperature for 5 min. Luminescence was then measured with a Bio-Tek Synergy HT plate reader (Winooski, VT). The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: human ACTH (1–24), [Nle<sup>4</sup>, D-Phe<sup>7</sup>] (NDP)- $\alpha$ -

MSH, and the dogfish (*S. acanthias*) melanocortins: ACTH (1–25),  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH (18).

#### Data analysis

All experimental treatments were performed in no less than triplicate and then corrected for control values, which were obtained by using transfected cells that were left unstimulated. In each of the assays, maximal activation levels were between 3 and 10 times the control levels. The values in the luciferase assays were normalized to the average readings for cells treated with 1  $\mu$ M human ACTH (1–24) (Figs. 4 and 5A and Supplemental Fig. 3) or 1  $\mu$ M dogfish ACTH (1–25) (Fig. 5B). Average values and SEM were graphed using Kaleidagraph software (Synergy Software, Reading, PA), and the  $EC_{50}$  value for each ligand was determined. The  $EC_{50}$  values were compared by using the Student's *t* test.

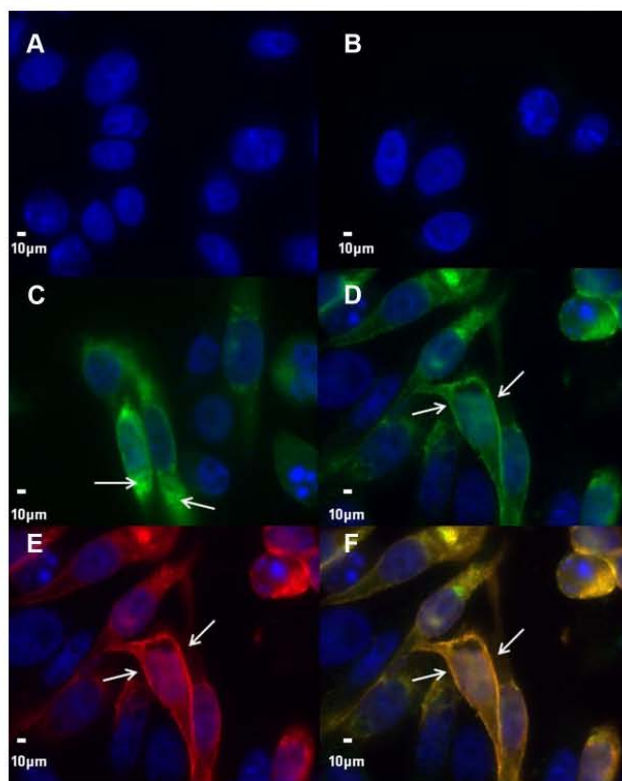
#### Maximum parsimony analysis

Maximum parsimony analysis was done using the branch and bound analysis algorithm (PAUP4.3). The following MCR amino acid sequences were analyzed: human MC1R (AAD41355.1), MC2R (AA067714.1), MC3R (AA072726.1), MC4R (AA092061.1), MC5R (NP\_005904.1), and zebrafish MC1R (AA162848.1), MC2R (NP\_775386.1), MC3R (AA024744.1), MC4R (AAL85494.1), and MC5R (NP\_775386.1). The sequence of *Lampetra fluviatilis* MCaR (lamprey; ABB36647.1) was used as the outgroup. The bootstrap values in Fig. 1 were derived from 1000 replications.

## Results

#### Immunofluorescence analysis of elephant shark MC2R expressed in CHO cells

To determine the parameters for expressing elephant shark MC2R in CHO cells, the V5-labeled *elephant shark mc2r* cDNA construct was transfected into CHO cells and V5 immunofluorescence was detected in permeabilized cells (data not shown). We also checked for V5 immunofluorescence in wells in which the transfected cells were not permeabilized. The operating assumption was that no immunofluorescence would be detected on the surface of the nonpermeabilized transfected CHO cells. However, as indicated in Fig. 2, V5 immunofluorescence was detected on the surface of the transfected CHO cells. Because CHO cells do not express an endogenous *mrp* gene (19), and no exogenous *mrp* cDNA was cotransfected with the *elephant shark mc2r* cDNA construct, it appears that the trafficking of the elephant shark MC2 receptor from the endoplasmic reticulum to the plasma membrane does not involve interaction with an MRAP. As an indication of the specificity of the V5 secondary antiserum, no immunofluorescence was detected when elephant shark MC2R-transfected CHO cells were incubated with the V5-specific secondary antibody alone (Fig. 3A). To demonstrate that our clone of CHO cells does not express endogenous *mrp*



**FIG. 3.** Immunofluorescence control experiments. A, To demonstrate the selectivity of the V5-specific secondary antibody, permeabilized elephant shark MC2R-transfected CHO cells were incubated with the secondary antiserum only, and no immunofluorescence was detected. B, To evaluate whether our clone of CHO cells express an endogenous MRAP, cells were transfected with a human V5-tagged *mc2r* cDNA alone and incubated with the V5 primary and secondary antibodies. For nonpermeabilized cells no immunofluorescence was observed on the plasma membrane. C, For permeabilized cells immunofluorescence was detected in subcellular compartments (white arrows). Hence, although the *hmc2r* construct was expressed in these CHO cells, human MC2R did not traffic to the plasma membrane. D, To demonstrate that exogenous MRAP can function in this clone of CHO cells, cells were cotransfected with V5-tagged *hmc2r* cDNA and a Flag-tagged mouse *mrapp1* cDNA, and nonpermeabilized cells were coincubated with the V5-specific antibody and the Flag-specific antibody. Incubation with the V5-specific secondary antibody revealed human MC2R immunofluorescence (green) on the plasma membrane (white arrows). E, Incubation of these same cells with the Flag-specific secondary antibody revealed mMRAP1 immunofluorescence (red) on the plasma membrane (white arrows). F, Overlaying of images D and E provides evidence for the colocalization (yellow) of human MC2R and mMRAP1. Images B, D, E, and F were acquired with the same setting and are displayed on the same intensity scale. Images A and C are displayed on distinct intensity scales. For all panels, blue demonstrates nuclei (DAPI). Space bar, 10  $\mu$ m.

genes, cells were transfected with a human *mc2r* cDNA, and no immunofluorescence was detected on the surface of nonpermeabilized cells (Fig. 3B). However, immunofluorescence was detected in permeabilized cells, which indicated that the *hmc2r* cDNA was expressed (Fig. 3C). Finally, to demonstrate that exogenous MRAP can be functionally expressed in our clone of CHO cells, cells

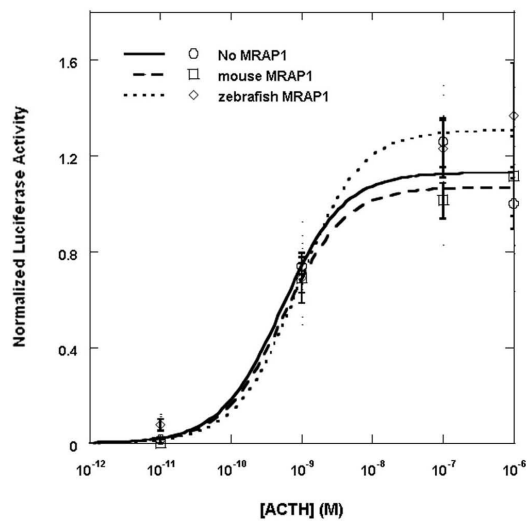
were cotransfected with a *hmc2r* cDNA and a mouse *mrapp1* cDNA. This experiment indicated that colocalization of the immunoreactive human MC2R and the mouse MRAP1 was apparent on the plasma membrane of nonpermeabilized cells (Fig. 3, D–F).

#### Coexpression of elephant shark MC2R with MRAP cDNA constructs

To determine whether coexpression with MRAP1 would affect the activation of elephant shark MC2R, CHO cells were first cotransfected with an *elephant shark mc2r* cDNA construct and either a mouse *mrapp1* (mMRAP1) cDNA construct or a zebrafish *mrapp1* (zfMRAP1) cDNA construct (Fig. 4). The mouse and zebrafish MRAP1 were used in this experiment due to the fact that a MRAP1 ortholog has not been found in the elephant shark genome database (10), yet mMRAP1 and zfMRAP1 can be used for functional activation of teleost MC2R (6, 8). The transfected cells were stimulated with human ACTH (1–24) in a dose-dependent manner (Fig. 4). Note that coexpression of elephant shark MC2R with either MRAP1 cDNA had no effect, either positive or negative, on activation. In this experiment the  $EC_{50}$  value for elephant shark MC2R expressed alone was  $5.2 \pm 1.0 \times 10^{-10}$  M, and in presence of mMRAP1 or zfMRAP1 the  $EC_{50}$  values were  $5.6 \pm 1.1 \times 10^{-10}$  M and  $8.9 \pm 1.9 \times 10^{-10}$  M, respectively. CHO cells were also cotransfected with the *elephant shark mc2r* cDNA construct and an *elephant shark mrapp2* cDNA construct and stimulated with human ACTH (1–24) (Supplemental Fig. 3). However, the presence of the elephant shark MRAP2 had no effect, either positive or negative, on the  $EC_{50}$  value.

#### Ligand selectivity of elephant shark MC2R

In a subsequent experiment, CHO cells were cotransfected with the *elephant shark mc2r* cDNA construct and stimulated with either human ACTH (1–24) or NDP-MSH. The operating assumption was that NDP-MSH



**FIG. 4.** Coexpression of the elephant shark MC2R with MRAP1. CHO cells were transfected with either the *elephant shark mc2 receptor* cDNA construct and the *cre/luc* cDNA construct only (solid curve) or with the mouse *mrp1* cDNA construct (dashed curve) or with the zebrafish *mrp1* cDNA construct (dotted curve), and 2 d after the transfection, wells containing  $1 \times 10^5$  cells were stimulated with human ACTH (1–24) at concentrations ranging from  $10^{-5}$  to  $10^{-12}$  M as described in *Materials and Methods*. Results are expressed as mean  $\pm$  SEM ( $n = 4$ ). The results were normalized to the average readings for cells treated with  $1 \mu\text{M}$  human ACTH (1–24).

would have no effect on the elephant shark MC2R ortholog. However, NDP-MSH activated the elephant shark MC2R in a dose-dependent manner (Fig. 5A). The  $\text{EC}_{50}$  value for the human ACTH (1–24)-stimulated cells was  $6.3 \pm 2.5 \times 10^{-10}$  M, and the  $\text{EC}_{50}$  value for the NDP-MSH-stimulated cells was  $1.3 \pm 0.6 \times 10^{-7}$  M. Although human ACTH (1–24) was clearly the more potent ligand ( $P < 0.05$ ), it is unprecedented for a MC2R ortholog to have any sensitivity for an MSH-sized ligand.

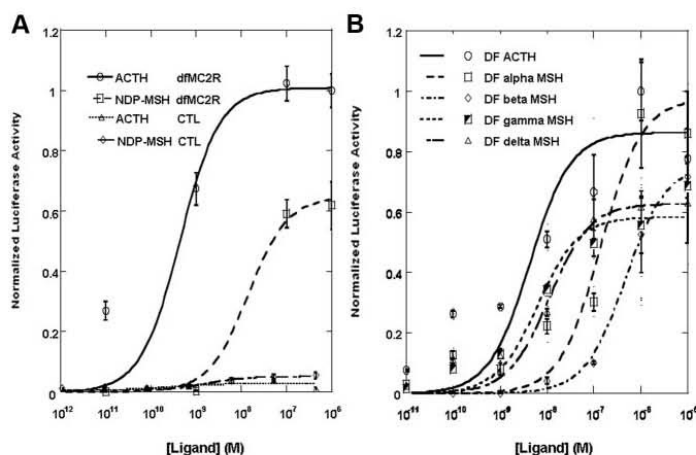
The potency of various cartilaginous fish melanocortins was also tested on CHO cells transfected with elephant shark MC2R. For this experiment the *S. acanthias* (dogfish) melanocortins ACTH (1–25),  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH (18) were tested at concentrations ranging from  $10^{-5}$  to  $10^{-11}$  M (Fig. 5B). The  $\text{EC}_{50}$  values for the dogfish melanocortins were as follows: ACTH (1–25),  $4.3 \pm 1.4 \times 10^{-9}$  M;  $\gamma$ -MSH,  $6.3 \pm 1.8 \times 10^{-9}$  M;  $\delta$ -MSH,  $1.1 \pm 0.2 \times 10^{-8}$  M;  $\alpha$ -MSH,  $1.4 \pm 0.5 \times 10^{-7}$  M; and  $\beta$ -MSH,  $4.7 \pm 1.4 \times 10^{-7}$  M. Although the  $\text{EC}_{50}$  values for ACTH (1–25),  $\gamma$ -MSH, and  $\delta$ -MSH were not statistically different, ACTH (1–25) was clearly a more potent ligand than either  $\alpha$ -MSH or  $\beta$ -MSH ( $P < 0.01$ ). The order of sensitivity for the dogfish melanocortins was ACTH (1–25) =  $\gamma$ -MSH =  $\delta$ -MSH >  $\alpha$ -MSH =  $\beta$ -MSH.

## Discussion

The distribution of melanocortin receptor paralogs and a MRAP2 paralog in extant cartilaginous fishes presents a number of unresolved questions. The model for the evolution and radiation of melanocortin receptor paralogs predicts that five MCR genes may have been present in the ancestral gnathostomes (10). Among the extant groups of gnathostomes (*i.e.* cartilaginous fishes, ray finned fishes, lobe finned fishes, and tetrapods), the presence of five MCR paralogs within individual species in each group has been confirmed with the exception of the cartilaginous fishes. Among these gnathostomes, cDNA were cloned from the genome of the elasmobranch, *S. acanthias*, which corresponded only to orthologs of MC3R, MC4R, and MC5R, respectively, (14, 15), whereas, an *in silico* analysis of the genome of the holocephalan, *C. milii* (elephant shark), revealed only the presence of orthologs of MC1R, MC2R, and MC3R, respectively. If in fact the ancestral gnathostomes had five paralogous MCR genes, then it would appear that gene loss has played a significant role in the selection of MCR paralogs in extant cartilaginous fishes.

Previous studies on the elasmobranch (sharks and rays) MCR (*i.e.* MC3R, MC4R, and MC5R) indicated that ACTH is more potent than any of the elasmobranch MSH (*i.e.*  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH) at activating these receptors (20, 21). It is known that corticotrophic cells of the anterior pituitary of elasmobranchs express the *pomc* gene, and these cells synthesize ACTH (18, 22). It is also known that melanotropic cells of the intermediate pituitary of elasmobranchs also express the *pomc* gene yet make the various MSHs (18, 23). Because elasmobranch MCR can be activated by either ACTH or the MSH, the mechanism for regulating the production of glucocorticoids by the interrenal glands of sharks and rays appears to be more complex than in the bony vertebrates. The receptor on the elasmobranch interrenal gland responsible for initiating glucocorticoid synthesis has not been identified. However, based on the current possibilities (*i.e.* MC3R, MC4R, or MC5R), activation of interrenal (HPA/I) cells cannot be regulated by a single melanocortin as seen in hypothalamus-pituitary-adrenal/hypothalamus-pituitary-interrenal axes of teleosts and tetrapods (3, 6, 24). Because agouti gene-related peptide (AGRP) have been detected in the genome of at least one cartilaginous fish (10), perhaps these polypeptides play a role in regulating glucocorticoid synthesis. Alternatively, another polypeptide/G protein-coupled receptor combination may regulate glucocorticoid synthesis in elasmobranchs. In this regard, a recent study on an angiotensin II receptor expressed by stingray head kidney cells (interrenal tissue) is relevant (25). Activation of this receptor will





**FIG. 5.** Ligand selectivity of the elephant shark MC2R. A, Functional expression of the elephant shark MC2R after stimulation with mammalian ligands. CHO cells were transfected with an elephant shark *mc2 receptor* cDNA construct and a *crelluc* cDNA construct. Two days after the transfection, wells containing  $1 \times 10^5$  cells were stimulated with either human ACTH (1–24) or NDP- $\alpha$ -MSH at concentrations ranging from  $10^{-6}$  to  $10^{-12}$  M as described in *Materials and Methods*. The reaction of nontransfected cells [control (CTL)] to these ligands is also shown. Results are expressed as mean  $\pm$  SEM ( $n = 3$ ). The results were normalized to the average readings for cells treated with  $1 \mu\text{M}$  human ACTH (1–24). B, Functional expression of the elephant shark MC2R after stimulation with dogfish melanocortins. CHO cells were transfected as described in A, and 2 d after the transfection, wells containing  $1 \times 10^5$  cells were stimulated with dogfish ACTH (1–25),  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, or  $\delta$ -MSH at concentrations ranging from  $10^{-5}$  to  $10^{-11}$  M as described in *Materials and Methods*. The results are expressed as mean  $\pm$  SEM ( $n = 3$ ). The results were normalized to the average readings for cells treated with  $1 \mu\text{M}$  dogfish ACTH (1–25).

result in glucocorticoid production. It would appear that multiple mechanisms may be present on elasmobranch interrenal cells to regulate the production of glucocorticoids. However, studies to delineate these mechanisms have not been done.

The current study has focused on the pharmacological properties of an MC2R ortholog in the genome of the holocephalan cartilaginous fish, *C. milii*. The identity of this receptor as a MC2R ortholog was based on a maximum likelihood analysis (10) and was supported by maximum parsimony analysis of a similar data set of MCR amino acid sequences (Fig. 1). However, the elephant shark MC2R ortholog has properties that are very different from either teleost or tetrapod MC2R orthologs. Immunofluorescence analyses and functional expression assays done in CHO cells indicated that the elephant shark MC2 receptor does not require MRAP for either trafficking to the plasma membrane (Fig. 2) or functional activation by human ACTH (1–24) (Fig. 4). Indeed, cotransfection with teleost or tetrapod MRAP1 or an elephant shark MRAP2 ortholog had no effect, either positive or negative, on the functional activation of the receptor (Fig. 4 and Supplemental Fig. 3). Although these data do not reveal a function for the elephant shark MRAP2 ortholog, collec-

tively these results indicate that the elephant shark MC2 receptor is an MRAP-independent MC2R ortholog.

This conclusion is not unprecedented. As presented in the introductory text, the working hypothesis for the evolution of the melanocortin receptor gene family (9, 10) is that the ancestral melanocortin gene emerged in an ancestral, and now extinct, protochordate lineage. Following the first chordate genome duplication event (1R), two paralogous MCR-like genes are predicted to have been present in the ancestral agnathans, and following the second chordate genome duplication event (2R), four paralogous melanocortin receptor genes are predicted to have been present in the ancestral gnathostomes. A local gene duplication of one of the paralogous melanocortin receptor genes in the ancestral gnathostome genome is predicted to account for the presence of a fifth paralogous melanocortin receptor gene in the stem gnathostome classes (*i.e.* the cartilaginous fishes, the ray finned fishes, and the lobe finned fishes and tetrapods)

(10). In support of this hypothesis, two paralogous MCR cDNA (MCa and MCb) have been characterized from the lamprey genome (26). Phylogenetic analyses indicate that MCa is a MC1R ortholog and MCb is a MC4R ortholog. The lamprey MCa receptor has been functionally expressed in human embryonic kidney-293 EBNA cells without the need for cotransfection with an exogenous *mrp* cDNA. Note that no MC2R orthologous gene has been detected in the lamprey genome.

With respect to the other cartilaginous fish melanocortin receptors (*i.e.* dogfish MC3R, MC4R, and MC5R), all of these receptors can be functionally expressed in heterologous mammalian cells without cotransfection with an exogenous *mrp* cDNA (14, 15, 21). When all these observations are taken collectively, two hypotheses emerge: 1) the MC2R gene most likely originated with the ancestral gnathostomes before the radiation of the three stem classes of gnathostomes; and 2) melanocortin receptors in agnathans and in the ancestral gnathostomes were MRAP independent. Based on these hypotheses, we predict that the MRAP-independent nature of the elephant shark MC2R was an ancestral feature rather than a derived trait.

Based on these assumptions, it would appear that dependence on MRAP for trafficking to the plasma mem-



brane and for functional activation of the MC2 receptor must have evolved after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes. Current understanding of the dependence of the MC2R on MRAP indicates that a complex forms between two MRAP homodimers and a MC2R homodimer (27). Although the MC2R-trafficking motif and functional activation motif on MRAP1 have been identified (28, 29), the corresponding contact site on the MC2 receptor for interaction with MRAP has not been determined for either teleost or tetrapod MC2R.

Another major difference between elephant shark MC2R and teleost and tetrapod MC2R is with respect to ligand selectivity. There is an extensive literature on the exclusive selectivity of the adrenal cortex (*i.e.* MC2R) receptor for ACTH and the inability of  $\alpha$ -MSH to activate this receptor (30) or even to act as a competitive inhibitor of ACTH binding to the receptor (31). Although ACTH (1–24) was a more potent stimulator of elephant shark MC2R than any of the MSH that were tested, the fact remains that the receptor could be activated by NDP-MSH or the dogfish fish MSH in a dose-dependent manner (Fig.

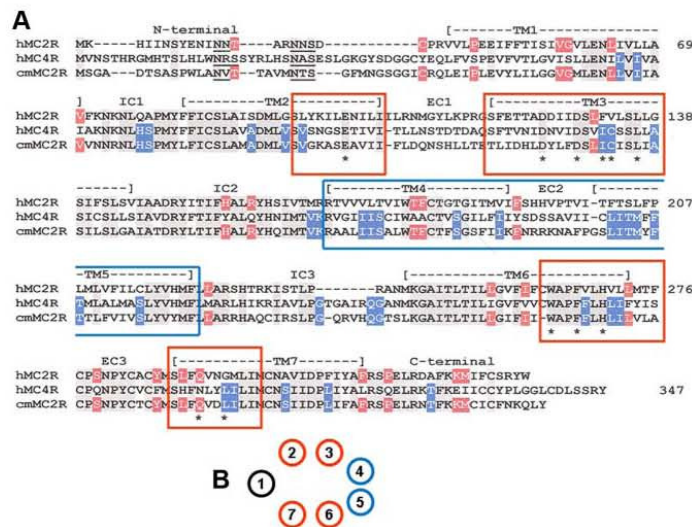
5, A and B). A similar preference for ACTH-sized ligands compared with MSH-sized ligands has been observed for the lamprey melanocortin-a (MCa) receptor (26), and the *S. acanthias* (dogfish) MC3R, MC4R, and MC5R (14, 15, 21).

Clearly the HFRW binding site on the elephant shark MC2R can accommodate either ACTH or the MSH. This is a feature that has been observed for teleost and tetrapod MC1R, MC3R, MC4R, and MC5R but not for a teleost or tetrapod MC2R ortholog. Some understanding of the ligand selectivity of the elephant shark MC2 receptor ortholog relative to the teleost and tetrapod MC2R orthologs may be apparent from a comparison of the amino acid sequences of human MC2R, human MC4R, and elephant shark MC2R (Fig. 6A).

The mammalian and cartilaginous fish lineages last shared a common ancestor well over 400 million years ago (32); hence, it would be assumed that the number of identical positions in the human MC2R sequences relative to the elephant shark MC2R sequence would be few. In fact, only 33% of the amino acid positions in this alignment are identical in all three sequences (Fig. 5A, residues shaded

gray). As indicated by the red-shaded amino acid positions, an additional 24 positions are identical in the human and elephant shark MC2R orthologs. However, there are 29 positions (shaded blue; Fig. 6A) that are identical for human MC4R and elephant shark MC2R. Clearly these positions did not influence the positioning of the elephant shark MC2R in the maximum parsimony analysis (Fig. 1) but may play a role in ligand selectivity. There are some unexpected similarities between human MC4R and elephant shark MC2R that are worth noting.

All melanocortin receptors have a binding site to accommodate the HFRW motif of the melanocortin ligands (for review see Ref. 3). Pogozheva *et al.* (33) used a computer-modeling strategy and a site-directed mutagenesis paradigm to identify 11 critical amino acids in human MC4R that form the HFRW binding site. These residues are marked with a star in Fig. 6A. Note that the human MC2R sequence has eight of these conserved amino acid positions. However, the elephant shark MC2R sequence has 10 of these conserved amino acid positions.



**FIG. 6.** Alignment of human MC2R, human MC4R, and elephant shark MC2R. A, The amino acid sequences for human MC2R, human MC4R, and elephant shark MC2R were aligned as described in the legend to Supplemental Fig. 1. Amino acid positions that were identical in all three sequences were shaded in gray. Amino acid positions that were found only in the human MC2R and elephant shark MC2R are shaded in red. Amino acid positions that are only found in the human MC4R and elephant shark MC2R are shaded in blue. The position of the amino acids required for the HFRW binding site of human MC4R (33) are marked with an asterisk. The portions of TM2, TM3, TM6, and TM7 that form the HFRW binding site on the human MC4R are in the red boxes for emphasis. TM4, EC2, and TM5 were placed in the box with the blue boundaries for emphasis. B, Cartoon representation of MC2R. The TM of MC2R are arranged in a barrel configuration. The site of the HFRW binding site is marked by the TM regions with a red border. The TM regions that could constitute the proposed location of the K/RKRR binding site are marked with a blue boundary.

The additional conserved amino acid positions are found in transmembrane region 3 at residues I<sup>132</sup> and C<sup>133</sup>. These two residues are also present at the same position in nearly every MC1R, MC3R, MC4R, and MC5R that has been characterized (16), including the two lamprey melanocortin receptor paralogs (26). Hence, it would be reasonable to assume that residues in transmembrane (TM) 2, TM3, TM6, and TM7 were also present in the ancestral vertebrate MCR. However, these two amino acid positions are not found in MC2R orthologs (Supplemental Fig. 2). Furthermore, ACTH binding to a teleost or tetrapod MC2R ortholog also requires accommodation of the K/RKRR motif (29), which is present in the ACTH sequence of gnathostomes (34).

Assuming that the transmembrane regions of melanocortin receptors are oriented as shown in Fig. 6B to form the HFRW binding site, then the likely site for the K/RKRR binding site would be in the TM4, extracellular loop 2 (EC2), or TM5 portion of the receptor. In these regions of the receptor (Fig. 6A, *blue box*), it appears that the elephant shark MC2R is more MC4R-like than MC2R-like. Amino acid substitutions in the TM4/EC2/TM5 during the early radiation of ancestral bony fishes could have altered the three dimensional shape of the receptor to create the K/RKRR binding site. Thus, studies on some of the older lineages of the bony fishes may shed light on these changes. However, the ability of the teleost and tetrapod MC2 orthologs to exclude the MSH indicates that these receptors must have a more dynamic activation mechanism, which involves a confirmation change when the appropriate ligand (*i.e.* ACTH) makes contact with the receptor. In this regard, characterization of the K/RKRR binding on MC2R orthologs will be important in deciphering the mechanism of activation.

Although the phylogenetic analyses indicate that the elephant shark MC2R is orthologous to the teleost and tetrapod MC2R, from a functional perspective, elephant shark MC2R has properties that are distinct from these bony vertebrate MC2R orthologs. It would appear that after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes, significant changes occurred to the ancestral bony fish MC2R ortholog, which have influenced the regulation of interrenal cells and adrenal cortex cells in teleosts and tetrapods.

## Acknowledgments

We thank Nancy Lorenzon for her assistance with the immunofluorescence analysis. The mouse MRAP1 cDNA construct was provided by Dr. Patricia Hinkle (Rochester University, Rochester, NY).

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This work was supported by Grant IOB 0516958 from the National Science Foundation (to R.M.D.).

Disclosure Summary: The authors have nothing to declare.

## References

- Hinkle PM, Sebag JA 2009 Structure and function of the melanocortin 2 receptor accessory protein. *Mol Cell Endocrinol* 300:25–31
- Webb TR, Clark AJL 2010 Minireview: the melanocortin 2 receptor accessory proteins. *Mol Endocrinol* 24:475–484
- Cone RD 2006 Studies on the physiological functions of the melanocortin system. *Endocr Rev* 27:736–749
- Mountjoy KG, Robbins LS, Mortrud MT, Cone RD 1992 The cloning of a family of genes that encode the melanocortin receptors. *Science* 257:1248–1251
- Kapas S, Cammas FM, Hinson JP, Clark AJL 1996 Agonist and receptor binding properties of adrenocorticotropin peptides using the cloned mouse adrenocorticotropin receptor expressed in a stably transfected HeLa cell line. *Endocrinology* 137:3291–3294
- Agulleiro MJ, Roy S, Sánchez E, Puchol S, Gallo-Payet N, Cerdá-Reverter JM 2010 Role of melanocortin receptor accessory proteins in the function of zebrafish melanocortin receptor type 2. *Mol Cell Endocrinol* 320:145–152
- Aluru N, Vijayan MM 2008 Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout. *Endocrinology* 149:4577–4588
- Liang L, Sebag JA, Egelston L, Serasinghe MN, Vee K, Reinick C, Angleson J, Hinkle PM, Dores RM 2011 Functional expression of frog and rainbow trout melanocortin 2 receptors using heterologous MRAP1s. *Gen Comp Endocrinol* 174:5–14
- Schiöth HB, Haitina T, Ling MK, Ringholm A, Fredriksson R, Cerdá-Reverter JM, Klovins J 2005 Evolutionary conservation of the structural, pharmacological, and genomic characteristics of the melanocortin receptor subtypes. *Peptides* 26:1886–1900
- Vastermark A, Schiöth HB 2011 The early origin of melanocortin receptors, agouti-related peptides, agouti signaling peptide, and melanocortin receptor accessory proteins with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus. *Eur J Pharmacol* 660:61–69
- Klovins J, Haitina T, Fridmanis D, Kilianova Z, Kapa I, Fredriksson R, Gallo-Payet N, Schiöth HB 2004 The melanocortin system in Fugu: Determination of POMC/AGRP/MCR gene repertoire and synteny, as well as pharmacology and anatomical distribution of the MCRs. *Mol Biol Evol* 21:563–579
- Ringholm A, Fredriksson R, Poliakova N, Yan YL, Postlethwait JH, Larhammar D, Schiöth HB 2002 One melanocortin 4 and two melanocortin 5 receptors from zebrafish show remarkable conservation in structure and pharmacology. *J Neurochem* 82:6–18
- Nelson JS 1994 *Fishes of the world*. 3rd ed. New York: John Wiley, Sons Press
- Ringholm A, Klovins J, Fredriksson R, Poliakova N, Larson ET, Kukkonen JP, Larhammar D, Schiöth HB 2003 Presence of melanocortin (MC4) receptor in spiny dogfish suggests an ancient vertebrate origin of central melanocortin system. *Eur J Biochem* 270:213–221
- Klovins J, Haitina T, Ringholm A, Löwgren M, Fridmanis D, Slaidina M, Stier S, Schiöth HB 2004 Cloning of two melanocortin (MC) receptors in spiny dogfish. *Eur J Biochem* 271:4320–4331
- Baron A, Vee K, Angleson J, Dores RM 2009 Modeling the evolu-

- tion of the MC2R and MCSR genes: studies on the cartilaginous fish, *Heterodontus francisci*. Gen Comp Endocrinol 161:13–19
17. Chepurny OG, Holz GG 2007 A novel cyclic adenosine monophosphate responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein coupled receptor drug discovery efforts. J Biomol Screen 12:740–746
  18. Amemiya Y, Takahashi A, Suzuki N, Sasayama Y, Kawachi H 1999 A newly characterized melanotropin in proopiomelanocortin in pituitaries of an elasmobranch, *Squalus acanthias*. Gen Comp Endocrinol 114:387–395
  19. Sebag JA, Hinkle PM 2007 Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. Proc Natl Acad Sci USA 104:20244–20249
  20. Haitina T, Takahashi A, Holméén L, Enberg J, Schiöth HB 2007 Further evidence for ancient role of ACTH peptides at melanocortin (MC) receptors; pharmacology of dogfish and lamprey peptides at dogfish MC receptors. Peptides 28:798–805
  21. Reinick CL, Liang L, Angleson JK, Dorés RM 2012 Functional expression of *Squalus acanthias* melanocortin 5 receptor in CHO cells, ligand selectivity and interaction with MRAP. Eur J Pharmacol 680:1–7
  22. Lowry PJ, Bennett HP, McMartin C 1974 The isolation and amino acid sequence of an adrenocorticotrophin from the pars distalis and a corticotrophin-like intermediate-lobe peptide from the neurointermediate lobe of the pituitary of the dogfish, *Squalus acanthias*. Biochem J 141:427–437
  23. Lowry PJ, Chadwick A 1970 Purification and amino acid sequence of melanocyte-stimulating hormone from the dogfish, *Squalus acanthias*. Biochem J 118:713–718
  24. Gantz I, Fong TM 2003. The melanocortin system. Am J Physiol Endocrinol Metab 284:E468–E474
  25. Evans AN, Rimoldi JM, Gadepalli SV, Nunez BS 2010 Adaptation of a corticosterone ELISA to demonstrate sequence-specific effects of angiotensin II peptides and C-type natriuretic peptide on 1  $\alpha$ -hydroxycorticosterone synthesis and steroidogenic mRNAs in the elasmobranch interrenal gland. J Steroid Biochem Mol Biol 120:149–154
  26. Haitina T, Klovins J, Takahashi A, Löwgren M, Ringholm A, Enberg J, Kawauchi H, Larson ET, Fredriksson R, Schiöth HB 2007 Functional characterization of two melanocortin (MC) receptors in lamprey showing orthology to the MC1 and MC4 receptor subtypes. BMC Evol Biol 7:101
  27. Cooray SN, Chung TT, Mazhar K, Szidonya L, Clark AJ 2011 Bioluminescence resonance energy transfer reveals the adrenocorticotropin (ACTH)-induced conformational change of the activated ACTH receptor complex in living cells. Endocrinology 152:495–502
  28. Sebag JA, Hinkle PM 2009 Regions of melanocortin w (MC2) receptor accessory protein necessary for dual topology and MC2 receptor trafficking and signaling. J Biol Chem 284:610–618
  29. Webb TR, Chan L, Cooray SN, Cheetham ME, Chapple JP, Clark AJ 2009 Distinct melanocortin 2 receptor accessory protein domains are required for melanocortin 2 receptor interaction and promotion of receptor trafficking. Endocrinology 150:720–726
  30. Schwyzler R 1977 ACTH: a short introductory review. Ann NY Acad Sci 297:3–26
  31. Buckley DI, Yamashiro D, Ramachandran J 1981 Synthesis of a corticotropin analogue that retains full biological activity after iodination. Endocrinology 109:5–9
  32. Carroll RL 1988 Vertebrate paleontology and evolution. New York: W. H. Freeman
  33. Pogozheva ID, Chai BX, Lomize AL, Fong TM, Weinberg DH, Nargund RP, Mulholland MW, Grantz I, Mosberg HI 2005 Interactions of human melanocortin 4 receptors with nonpeptide and peptide agonists. Biochemistry 44:11329–11341
  34. Dorés RM, Lecaude S 2005 Trends in the evolution of the proopiomelanocortin gene. Gen Comp Endocrinol 142:81–93
  35. Dorés RM, Rubin DA, Quinn TW 1996 Minireview: is it possible to construct phylogenetic trees using polypeptide hormone sequences. Gen Comp Endocrinol 103:1–12

## **APPENDIX 4**





## Molecular and Cellular Pharmacology

Functional expression of *Squalus acanthias* melanocortin-5 receptor in CHO cells: Ligand selectivity and interaction with MRAPChristina L. Reinick, Liang Liang, Josepha K. Angleson, Robert M. Does\*<sup>\*</sup>

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## ARTICLE INFO

## Article history:

Received 16 August 2011

Received in revised form 18 January 2012

Accepted 21 January 2012

Available online 31 January 2012

## Keywords:

*Squalus acanthias*Melanocortin MC<sub>5</sub> receptor

CHO cells

ACTH

 $\alpha$ -MSH

MRAP

## ABSTRACT

The melanocortin-5 receptor (MC<sub>5</sub>) of the dogfish *Squalus acanthias* (SacMC<sub>5</sub> receptor) can be functionally expressed in CHO cells in the absence of the co-expression of an exogenous MRAP cDNA. Both human ACTH(1–24) and dogfish ACTH(1–25) were much better stimulators of the SacMC<sub>5</sub> receptor than any of the mammalian or dogfish MSH ligands that were tested. The order of ligand selectivity for the dogfish melanocortins was ACTH(1–25) >  $\alpha$ -MSH >  $\gamma$ -MSH =  $\delta$ -MSH >  $\beta$ -MSH. Unlike mammalian MC<sub>5</sub> receptors, the functional expression of the SacMC<sub>5</sub> receptor was not negatively impacted when the receptor was co-expressed with a cartilaginous fish (*Callorhynchus milii*) MRAP2 cDNA. However, co-expression with either mouse mMRAP1 or zebrafish zMRAP1 increased the sensitivity of SacMC<sub>5</sub> receptor for hACTH(1–24) by at least one order of magnitude. Hence, SacMC<sub>5</sub> receptor has the potential to interact with MRAP1 orthologs and in this regard behaved more like a melanocortin MC<sub>3</sub> receptor ortholog than a melanocortin MC<sub>5</sub> receptor ortholog. These observations are discussed in light of the evolution of the melanocortin receptor gene family in cartilaginous fish, and the physiological implications of these observations are considered.

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## 1. Introduction

The functional expression of melanocortin receptors in heterologous mammalian cells is a useful procedure for analyzing the ligand selectivity and trafficking requirements of these G-protein coupled receptors. In mammals there are five receptors in the melanocortin receptor gene family (MC<sub>1</sub> receptor, MC<sub>2</sub> receptor, MC<sub>3</sub> receptor, MC<sub>4</sub> receptor, and MC<sub>5</sub> receptor) and these receptors are activated with varying degrees of efficacy by the melanocortin peptides (ACTH,  $\alpha$ -MSH,  $\beta$ -MSH, and  $\gamma$ -MSH) (Cone, 2006). Orthologs of both mammalian and fish MC<sub>1</sub> receptor, MC<sub>3</sub> receptor, MC<sub>4</sub> receptor, and MC<sub>5</sub> receptor have been functionally expressed in mammalian HEK293 cells (Agulleiro et al., 2010; Cone, 2006; Klovins et al., 2004a; Ringholm et al., 2002). Hence, following the cloning of orthologs of MC<sub>3</sub> receptor, MC<sub>4</sub> receptor, and MC<sub>5</sub> receptor from the genome of the cartilaginous fish, *Squalus acanthias* (Sac), cDNA constructs of dogfish melanocortin receptors were individually transfected in HEK293 cells (Klovins et al., 2004b; Ringholm et al., 2003). Although this approach resolved the ligand selectivity properties of the SacMC<sub>3</sub> receptor and the SacMC<sub>4</sub> receptor (Haitina et al., 2007a; Klovins et al., 2004b; Ringholm et al., 2003), the SacMC<sub>5</sub> receptor-transfected HEK293 cells could not be stimulated by any melanocortin ligand (Ringholm et al., 2003). Subsequent transfection of a SacMC<sub>5</sub> receptor-EGFP fusion protein cDNA construct

into HEK293 cells revealed that the fusion protein did not move to the plasma membrane, but was retained in intracellular compartments (Klovins et al., 2004b).

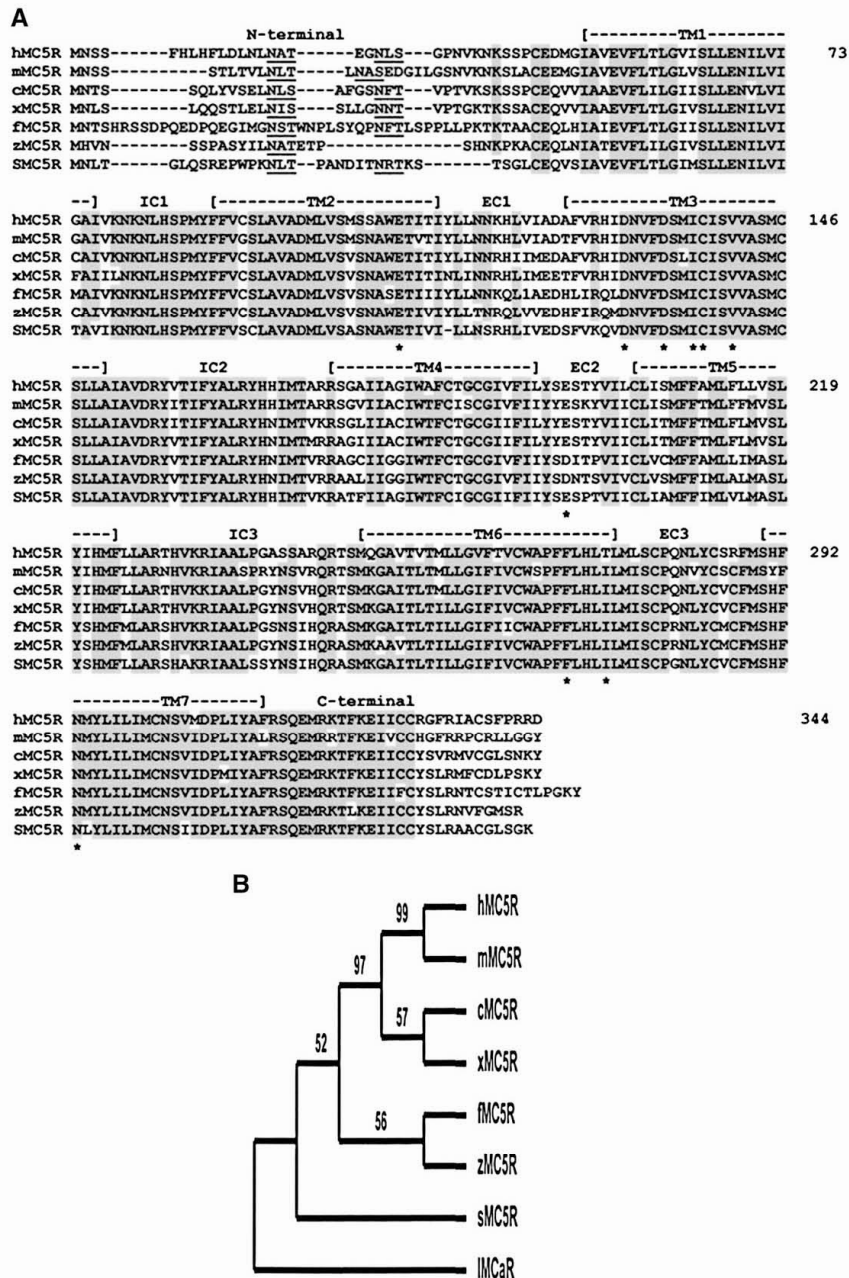
The inability of the SacMC<sub>5</sub> receptor to move from the endoplasmic reticulum to the plasma membrane in HEK293 cells may have been the result of improper folding of the SacMC<sub>5</sub> receptor in the endoplasmic reticulum (Tan et al., 2003). However, a comparison of the SacMC<sub>5</sub> receptor amino acid sequence to other vertebrate MC<sub>5</sub> receptor sequences (Fig. 1A) does not immediately reveal any obvious alterations in the primary sequence of the SacMC<sub>5</sub> receptor that might have induced improper folding of this receptor, and maximum parsimony analysis (Fig. 1B) indicated that SacMC<sub>5</sub> receptor is the sister sequence to a clade of bony vertebrate MC<sub>5</sub> receptor sequences.

It is possible that the functional expression of the SacMC<sub>5</sub> receptor in HEK293 cells may require interaction with an accessory protein to facilitate trafficking to the plasma membrane in a manner analogous to the interaction between mammalian and bony fish melanocortin-2 receptors (MC<sub>2</sub> receptors) and melanocortin receptor accessory protein (MRAP) (Agulleiro et al., 2010; Metherell et al., 2005; Roy et al., 2007; Sebag and Hinkle, 2007, 2009a; Webb et al., 2009). However, studies on the interaction between mammalian MC<sub>5</sub> receptor and MRAP indicated that trafficking of this melanocortin receptor to the plasma membrane is actually inhibited following co-transfection with either MRAP 1 or MRAP2 (Sebag and Hinkle, 2009b).

Given these various possibilities, the following study was done to determine whether the SacMC<sub>5</sub> receptor could be functionally expressed in Chinese Hamster Ovary (CHO) cells. The recent discovery of an MRAP2 ortholog in the genome of the cartilaginous fish,

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**Fig. 1.** Alignment of vertebrate MC<sub>5</sub> receptor amino acid sequences. A) The amino acid sequences of *Homo sapiens* (hMC5R; NP\_005904.1), *Mus musculus* (mMC5R; AA100721.1), *Gallus gallus* (cMC5R; NP\_001026186.1), *Xenopus tropicalis* (xMC5R; NP\_001096392.1), *Takifugu rubripes* (fMC5R; AA024750.1), *Danio rerio*, (zMC5R; NP\_775386.1) and *Squalus acanthias* (sMC5R; AAS67890) were aligned as described previously (Dores et al., 1996). Amino acid positions shaded in gray were identical in 86% of the taxa. N-linked glycosylation sites are underlined. A (\*) denotes an amino acid position that is in the proposed HFRW binding site of the receptor (Pogozheva et al., 2005). Abbreviations: TM—transmembrane spanning domain; EC—extracellular loop; IC—intracellular loop. B) Maximum parsimony analysis was done using the exhaustive analysis algorithm (PAUP 4.3). The sequence of *Lampetra fluviatilis* MC<sub>5</sub> receptor (IMCaR; ABB36647.1) was used as the outgroup. The bootstrap values in B were derived from 1000 replications.



*Callorhynchus milii* (Cm) (Vastermark and Schiöth, 2011) provided the opportunity to test the hypothesis that co-transfection of a SacMC<sub>5</sub> receptor cDNA with a CmMRAP2 cDNA would influence the functional expression of the SacMC<sub>5</sub> receptor in CHO cells. Finally, once the conditions for expressing the SacMC<sub>5</sub> receptor in CHO cells were resolved, the primary objective of this study was to determine the ligand selectivity of the SacMC<sub>5</sub> receptor using both mammalian and dogfish melano-cortin ligands.

## 2. Materials and methods

### 2.1. MC<sub>5</sub> receptor and MRAP constructs

Spiny dogfish (*S. acanthias*) MC<sub>5</sub> receptor, accession number AY562212, was synthesized by GenScript (Piscataway, NJ) with a V-5 epitope tag, and the cDNA was inserted into a pcDNA3.1 vector. Mouse (*Mus musculus*) mMRAP1, accession number NM\_029844, zebrafish (*Danio rerio*) zbMRAP1, accession number XR\_117835, and elephant shark (*C. milii*) CmMRAP2, accession number BR000861, were also synthesized by GenScript with a FLAG epitope tag, respectively, and each cDNA was separately inserted into pcDNA3.1 vectors.

### 2.2. Tissue culture

The experiments were all done in transiently transfected CHO cells (ATCC, Manassas, VA). The CHO cells were grown at 37 °C in a humidified 5% CO<sub>2</sub> incubator in Kaighn's Modification of Ham's F-12 (2 mM glutamine, 1500 mg/l bicarbonate; ATCC, Manassas, VA) with 10% fetal bovine serum, 5 ml pen/strep, and 1 ml normocin.

### 2.3. Immunocytochemistry

CHO cells were grown in 2-well chamber slides (1 × 10<sup>5</sup> cells/well) for 24 h prior to transfection. The cells were transfected with 1 µg of dogfish mc<sub>5</sub> receptor cDNA construct using lipofectamine 2000 (Invitrogen; www.invitrogen.com) and Opti-MEM (Cellgro, Manassas,

VA). Twenty-four hours post-transfection, the cells were fixed with 4% paraformaldehyde for 15 min then either permeabilized using 0.3% triton for 10 min (Fig. 2A) or left unpermeabilized (Fig. 2C). The primary antibody was diluted 1:100 in PBS + 1% BSA solution. A mouse monoclonal anti-V5 antibody was used to detect SacMC5R. The cells were incubated with the primary antibodies for 1 h at 37 °C. After a wash step, the cells were incubated with secondary antibody for 45 min at 37 °C. A donkey anti-mouse antibody linked to Alexa-Fluor488 was used to visualize SacMC5R (green). The secondary antibody was diluted 1:400 (donkey anti-mouse) in PBS + 1% BSA. Cover slips were mounted onto slides using Vectashield (Vector Lab, Burlingame, CA), and nuclei were stained with DAPI (blue). The controls for this experiment were the absence of staining in non-transfected cells following incubation with the primary and secondary antibodies (Fig. 2B), and the absence of staining in transfected cells following separate incubation with the secondary antiserum (Fig. 2D). Images were obtained using a 100× oil immersion objective (Zeiss Plan-NEOFUAR) with a fluorescence microscope equipped with a Hamamatsu digital camera. All images were analyzed using SlideBook (www.slidebook.com) software.

### 2.4. Functional expression assays

CHO cells were transfected with 2 µg each of SacMC<sub>5</sub> receptor, MRAP, and CRE-Luc reporter plasmid (Chepurny and Holz, 2007) using a Cell Line Nucleofector Kit (Amaxa, Inc.; www.lonza.com) with solution T and program U-023. Experiments were also done in which CHO cells were only transfected with SacMC<sub>5</sub> receptor and the CRE-Luc reporter plasmid. The cells were then plated on a white, 96-well plate at a density of 1 × 10<sup>5</sup> cells per well. Forty-eight hours after transfection, cells were stimulated with the appropriate ligands in serum-free CHO media for 4 h at 37 °C at concentrations ranging from 10<sup>−5</sup> M to 10<sup>−10</sup> M. After a four hour incubation period, 100 µl of Bright-Glo luciferase assay reagent (Promega Inc., Madison, WI) was applied to each well and incubated at room temperature for 5 min. Luminescence was then measured with a Bio-Tek Synergy HT

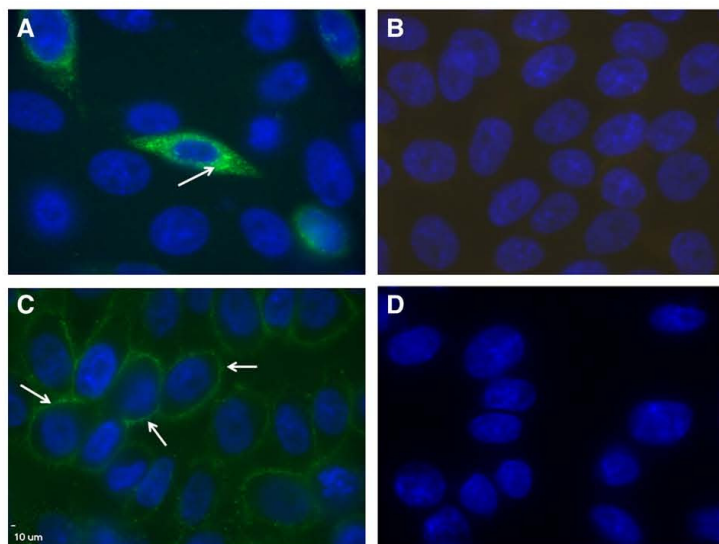


Fig. 2. CHO cells expressing the SacMC<sub>5</sub> receptor. A) The V5 tagged SacMC<sub>5</sub> receptor is seen in the cytoplasm of permeabilized cells (arrow). B) Non-transfected CHO cells shown no reaction when incubated with the primary and secondary antisera. C) The V5 tagged SacMC<sub>5</sub> receptor was detected on the cell surface of unpermeabilized CHO cells (white arrows). D) Transfected CHO cells incubated with only the secondary antiserum did not show any reaction. Green = SacMC<sub>5</sub> receptor (Alexa-Fluor488). Blue = nuclei (DAPI). Space bar = 10 µm.

plate reader (Winooski, VT). The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: human ACTH(1–24), NDP- $\alpha$ -MSH, and the dogfish melanocortins: ACTH(1–25),  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH (Amemiya et al., 1999).

### 2.5. Data analysis

All experimental treatments were performed in no less than triplicate, and then corrected for control values, which were obtained by using transfected cells that were left unstimulated. In each of the assays, maximal activation levels were between three to ten times the control level. Corrected values were normalized to the average readings for cells treated with 1  $\mu$ M ACTH (for the luciferase assay). Average values and standard errors of the mean were graphed using KaleidaGraph software ([www.synergy.com](http://www.synergy.com)), and the EC<sub>50</sub> value for each ligand was determined. The curves were not constrained through the 0/0 origin. The EC<sub>50</sub> values were compared by using the student's t-test.

### 2.6. Maximum parsimony analysis

Maximum parsimony analysis was done using the exhaustive analysis algorithm (PAUP 4.3, [paup.csit.fsu.edu](http://paup.csit.fsu.edu)). The following MC<sub>5</sub> receptor amino acid sequences were analyzed: *Homo sapiens* (human; NP\_005904.1), *M. musculus* (mouse; AA100721.1), *Gallus gallus* (chicken; NP\_001026186.1), *Xenopus tropicalis* (NP\_001096392.1), *Takifugu rubripes* (fugu fish; AA024750.1), *D. rerio*; (zebrafish; NP\_775386.1), and *S. acanthias* (dogfish; AA567890). The sequence of *Lampetra fluviatilis* (lamprey; ABB36647.1) MCaR was used as the outgroup. The bootstrap values in Fig. 1B were derived from 1000 replications.

## 3. Results

### 3.1. Immunocytochemical staining of CHO cells expressing the SacMC<sub>5</sub> receptor

In order to evaluate the expression of the SacMC<sub>5</sub> receptor in CHO cells, the cells were transfected with a *sacmc5* receptor cDNA construct that encoded a V5 epitope tag located at the N-terminal of the receptor. Two days post-transfection, as expected, permeabilized cells showed a positive reaction when incubated with the V5 epitope antibody (Fig. 2A). What was not expected was that immunocytochemical staining of a parallel well of unpermeabilized transfected cells showed a positive reaction on the surface of the transfected cells when incubated with the V5 epitope antibody (Fig. 2C). In the controls for this experiment, non-transfected cells showed no reaction when incubated with the primary and secondary antibodies (Fig. 2B), as well as transfected cells incubated with the secondary antibody alone (Fig. 2D).

### 3.2. Ligand selectivity of the SacMC<sub>5</sub> receptor

Once it was established that the SacMC<sub>5</sub> receptor was trafficking to the plasma membrane of CHO cells, it seemed expeditious to determine whether the SacMC<sub>5</sub> receptor could be activated by melanocortin ligands. For these experiments, the *sacmc5* receptor cDNA and the *cre-luc* cAMP reporter cDNA were co-transfected into CHO cells, and two days post-transfection cells were stimulated with either human ACTH(1–24) or mammalian NDP- $\alpha$ -MSH at concentrations ranging from 10<sup>–5</sup> to 10<sup>–10</sup> M (Fig. 3A). Both mammalian ligands stimulated the transfected CHO cells in a dose dependent manner. The EC<sub>50</sub> for hACTH(1–24) was  $5.4 \pm 1.1 \times 10^{-8}$  M as compared to an EC<sub>50</sub> for NDP- $\alpha$ -MSH of  $9.8 \pm 2.3 \times 10^{-8}$  M. While it appeared that hACTH(1–24) might be a more potent ligand than NDP- $\alpha$ -MSH, stimulation by these two ligands was not statistically different ( $P = 0.8$ ). Next the SacMC<sub>5</sub> receptor transfected CHO cells were stimulated

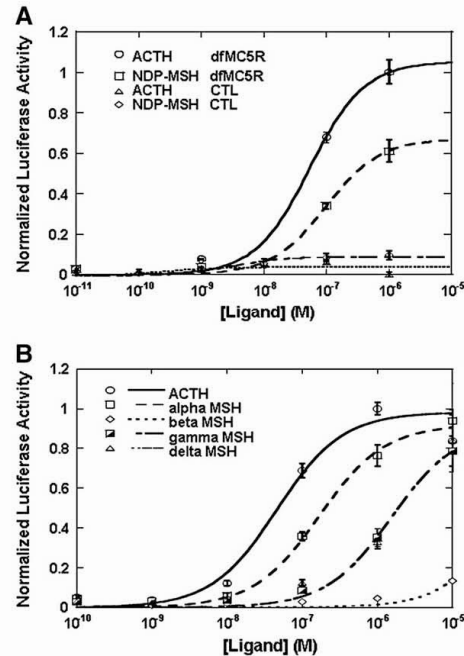


Fig. 3. Ligand selectivity of the SacMC<sub>5</sub> receptor. A) Functional expression of the SacMC<sub>5</sub> receptor after stimulation with mammalian ligands. CHO cells were transfected with a *sacmc5* receptor cDNA construct and a *cre-luc* cDNA construct. Two days post-transfection, wells containing  $1 \times 10^5$  cells were stimulated with either hACTH(1–24) or NDP- $\alpha$ -MSH at concentrations ranging from 10<sup>–5</sup> M to 10<sup>–11</sup> M as described in Materials and methods. The reaction of non-transfected cells (control = CTL) to these ligands is shown. Results are expressed as mean  $\pm$  S.E.M.;  $n = 3$ . B) Functional expression of the SacMC<sub>5</sub> receptor after stimulation with dogfish melanocortins. CHO cells were transfected as described in (A), and two-days post-transfection wells containing  $1 \times 10^5$  cells were stimulated with either dogfish ACTH(1–25),  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH or  $\delta$ -MSH as described in Materials and methods. Results are expressed as mean  $\pm$  S.E.M.;  $n = 3$ .

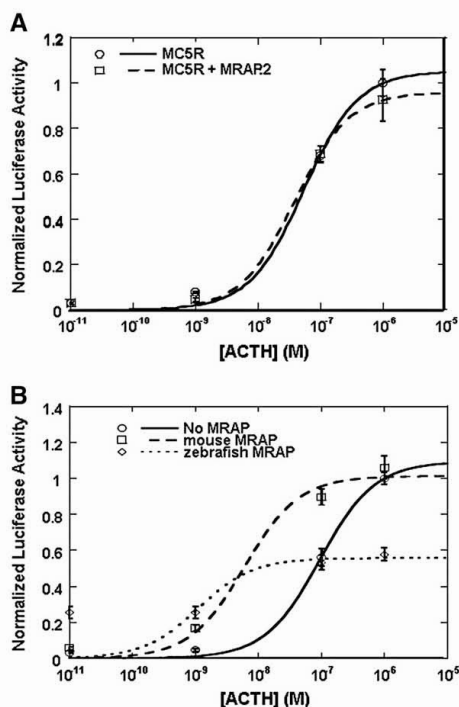
with dogfish melanocortins at concentrations ranging from 10<sup>–5</sup> to 10<sup>–10</sup> M (Fig. 3B). In these experiments, dogfish ACTH(1–25) was the most potent ligand, whereas dogfish  $\beta$ -MSH induced minimal activation only at a concentration of 10<sup>–5</sup> M. The EC<sub>50</sub> values for each ligand were: ACTH(1–25) ( $4.6 \pm 0.9 \times 10^{-8}$  M),  $\alpha$ -MSH ( $1.8 \pm 0.3 \times 10^{-7}$  M),  $\gamma$ -MSH ( $1.5 \pm 0.3 \times 10^{-6}$  M), and  $\delta$ -MSH ( $2.0 \pm 0.3 \times 10^{-6}$  M), and the P value for ACTH(1–25) potency relative to the potency of each MSH was 0.05 ( $\alpha$ -MSH), 0.005 ( $\gamma$ -MSH), and 0.05 ( $\delta$ -MSH). The order of sensitivity for the dogfish melanocortins was ACTH(1–25) >  $\alpha$ -MSH >  $\gamma$ -MSH =  $\delta$ -MSH >  $\beta$ -MSH.

### 3.3. Sac MC<sub>5</sub> receptor co-expression with MRAPs

Since SacMC<sub>5</sub> receptor could be functionally expressed in CHO cells in the apparent absence of an accessory protein(s), we then tested the hypothesis that the functional activation of the SacMC<sub>5</sub> receptor would be inhibited by co-expression with MRAPs in a manner analogous to interaction between mammalian MC<sub>5</sub> receptors and the MRAPs (Webb and Clark, 2010). In the first experiment, the effect of a *C. milli* MRAP2 (CmMRAP2) on the functional expression of the SacMC<sub>5</sub> receptor in CHO cells was tested (Fig. 4A). However, this MRAP had no effect, either positive or negative, on the activation of the SacMC<sub>5</sub> receptor.

The next experiment was to determine whether the activation of the SacMC<sub>5</sub> receptor might be affected by co-expression with MRAP1. For this experiment the *sacmc5* receptor cDNA construct was





**Fig. 4.** Co expression of the SacMC<sub>5</sub> receptor with MRAPs. A) CHO cells were transfected with either the *sacmc5* receptor cDNA construct and the *cre/luc* cDNA construct (solid curve), or the *sacmc5* receptor cDNA construct, the *cre/luc* cDNA construct and the *Callorhynchus milii* (Cm) *mrp2* cDNA construct (dashed curve), and two days post-transfection wells containing  $1 \times 10^5$  cells were stimulated with hACTH(1–24) at concentrations ranging from  $10^{-5}$  M to  $10^{-11}$  M as described in **Materials and methods**. Results are expressed as mean  $\pm$  S.E.M.;  $n=4$ . B) Three sets of CHO cells were transfected with either a) *sacmc5* receptor cDNA and *cre/luc* cDNA (solid curve); b) *sacmc5* receptor cDNA, *cre/luc* cDNA and mouse *mrap1* cDNA (dashed curve); or c) *sacmc5* receptor cDNA, *cre/luc* cDNA, and zebrafish *zfmrap1* (dotted curve), and two days post-transfection wells containing  $1 \times 10^5$  cells were stimulated with hACTH(1–24) at concentrations ranging from  $10^{-5}$  M to  $10^{-11}$  M as described in **Materials and methods**. Results are expressed as mean  $\pm$  S.E.M.;  $n=8$ .

either: a) expressed alone in CHO cells; b) co-expressed with mouse MRAP1 (mMRAP1); or c) co-expressed with zebrafish MRAP1 (zfMRAP1). As indicated in **Fig. 4B**, rather than inhibiting the activation of the SacMC<sub>5</sub> receptor, the vertebrate MRAPs significantly improved the sensitivity of the receptor for hACTH(1–24) ( $P=0.005$ ). While the EC<sub>50</sub> value for the dose response curve when the SacMC<sub>5</sub> receptor was expressed alone was  $9.4 \pm 1.4 \times 10^{-8}$  M; when the SacMC<sub>5</sub> receptor was co-expressed with mouse MRAP1 the EC<sub>50</sub> value was  $6.0 \pm 2.0 \times 10^{-9}$  M, and when the dogfish receptor was co-expressed with zebrafish MRAP1 the EC<sub>50</sub> value was  $1.1 \pm 0.5 \times 10^{-9}$  M. **Fig. 4B** also shows that while co-transfection of the SacMC<sub>5</sub> receptor with zebrafish MRAP1 led to a lower EC<sub>50</sub> value, it ultimately resulted in a significantly lower maximum activation value than the SacMC<sub>5</sub> receptor co-expressed with mouse MRAP1 or when the SacMC<sub>5</sub> receptor was expressed alone.

#### 4. Discussion

Based on the results of the current study it would appear that the inability to functionally express the SacMC<sub>5</sub> receptor in HEK293 may have been an intrinsic feature in the primary sequence of the SacMC<sub>5</sub> receptor construct that affected the folding of the receptor in the

endoplasmic reticulum of HEK293 cells. Since several melanocortin receptors, from a rather wide range of vertebrates, have been functionally expressed in HEK293 cells (Agulleiro et al., 2010; Cone, 2006; Klovins et al., 2004a; Ling et al., 2004; Ringholm et al., 2002), this is possibly an anomaly unique to the SacMC<sub>5</sub> receptor.

#### 4.1. Evolution of MC<sub>5</sub> receptor

The immunocytochemical analysis and the ligand selectivity studies indicate that the SacMC<sub>5</sub> receptor can be functionally expressed in a heterologous cell line (CHO cells) without co-expression of an exogenous MRAP cDNA; a feature common to teleost and mammalian MC<sub>5</sub> receptors (Cone, 2006; Schiöth et al., 2005). Since the origin of the MC<sub>5</sub> receptor gene and the other paralogous genes in the melanocortin receptor family appears to be the result of two predicted genome duplication events that occurred during the evolution of the chordates (Holland et al., 1994; Lundin, 1993; Ohno et al., 1968), and at least one local gene duplication that most likely occurred early in the evolution of the ancestral gnathostomes (Baron et al., 2009; Klovins et al., 2004a; Schiöth et al., 2005), this scenario would account for the presence of the MC<sub>5</sub> receptor ortholog in all of the stem gnathostome lineages (e.g., cartilaginous fishes, ray-finned fishes, and lobe finned fishes; for reviews see Vastermark and Schiöth, 2011; Veo et al., 2011). During the radiation of the stem gnathostomes some of the functional properties of the MC<sub>5</sub> receptor orthologs have diverged, and this study has focused on two aspects of this functional evolution; ligand selectivity and interaction with MRAP.

#### 4.2. Phylogeny of melanocortin receptor ligand selectivity

With respect to ligand selectivity, recent studies on the two lamprey melanocortin receptors and on the MC<sub>3</sub> receptor and MC<sub>4</sub> receptor orthologs present in the dogfish genome indicate that these melanocortin receptors have a higher affinity for ACTH than for the MSHs (Haitina et al., 2007a). These observations have led to the conclusion that ACTH might have been the primary ligand for the ancestral melanocortin receptors (Haitina et al., 2007b). The results of this study indicate that the SacMC<sub>5</sub> receptor has retained this apparent ancestral feature. As shown in **Fig. 3**, the SacMC<sub>5</sub> receptor has a higher sensitivity for ACTH [either human ACTH(1–24) or dogfish ACTH(1–25)] than any of the dogfish MSHs. In addition, the order of ligand selectivity for the dogfish melanocortins was ACTH(1–25) >  $\alpha$ -MSH >  $\gamma$ -MSH =  $\delta$ -MSH >  $\beta$ -MSH; the same ligand selectivity profile observed for the SacMC<sub>3</sub> receptor and the SacMC<sub>4</sub> receptor functionally expressed in HEK293 cells (Haitina et al., 2007a). The SacMC<sub>3</sub> receptor and the SacMC<sub>4</sub> receptor also have a higher affinity for  $\delta$ -MSH than  $\beta$ -MSH, and  $\delta$ -MSH was a more potent ligand for the SacMC<sub>5</sub> receptor as compared to  $\beta$ -MSH.

Finally these observations on non-mammalian MC<sub>5</sub> receptor orthologs are in sharp contrast to the ligand selectivity of mammalian MC<sub>5</sub> receptor orthologs. For mammalian MC<sub>5</sub> receptors the order of ligand selectivity is  $\alpha$ -MSH > ACTH >  $\beta$ -MSH >  $\gamma$ -MSH (Cone, 2006). All of the melanocortin peptides activate the melanocortin receptors through contact with the HFRW motif present in these ligands (Cone, 2006), and the critical amino acids in TM2, TM3, TM6, and TM7 that form the proposed HFRW binding site (Pogozheva et al., 2005) are rigorously conserved in the MC<sub>5</sub> receptor sequences presented in **Fig. 1**. Thus, there must be some tertiary feature in non-mammalian MC<sub>5</sub> receptors to allow ACTH to fit into the HFRW binding site with greater efficacy than  $\alpha$ -MSH.

#### 4.3. Phylogeny of melanocortin receptor interaction with MRAP

Another distinctive feature of mammalian MC<sub>5</sub> receptor is the interaction with the MRAPs. The MRAPs (MRAP1 or MRAP2) facilitate the trafficking of mammalian MC<sub>5</sub> receptor to the plasma membrane.

In addition, MRAP1 is required for full activation of the MC<sub>2</sub> receptor following an ACTH binding event (Hinkle and Sebag, 2009; Webb and Clark, 2010). MRAPs have an opposite effect on mammalian MC<sub>5</sub> receptors. For this receptor, the MRAPs interfere with MC<sub>5</sub> receptor dimerization and trafficking of the receptor to the plasma membrane (Chan et al., 2009; Sebag and Hinkle, 2009b). Although the MC<sub>2</sub> receptor is the predominant melanocortin receptor on mammalian adrenocortical cells (Cone, 2006; Mountjoy et al., 1992), the MC<sub>5</sub> receptor is also expressed in adrenocortical cells of some mammals (van der Kraan et al., 1998). It would appear that the co-expression of MRAP1 and MRAP2 in adrenocortical cells selectively enhances the functional expression of the MC<sub>2</sub> receptor, and inhibit the functional expression of the MC<sub>5</sub> receptor to ensure that adrenocortical cells are only stimulated by ACTH, but not by any of the other melanocortins (Hinkle and Sebag, 2009; Metherell et al., 2005; Roy et al., 2007; Webb and Clark, 2010).

Interactions between the MC<sub>5</sub> receptor and the MRAPs have only been analyzed for some mammalian MC<sub>5</sub> receptor orthologs. As a result, it is not known at which point or points in the phylogeny of the vertebrates the interaction between the MC<sub>5</sub> receptor and the MRAPs emerged. Currently there have been no reports on the characterization of MRAP orthologs from the genome of the dogfish. However, a MRAP2 ortholog had been identified in the genome of the holocephalan cartilaginous fish, *C. milii* (Cm) (Vastermark and Schiöth, 2011). Since an MRAP2 ortholog has also been detected in the genome of the lamprey *Petromyzon marinus* (Vastermark and Schiöth, 2011), it is likely that the genome of the dogfish, might also have an *mrp2* gene. We tested the hypothesis that co-expression of the SacMC<sub>5</sub> receptor with CmMRAP2 would result in a relationship analogous to the mammalian MC<sub>5</sub> receptor/MRAP interaction. As indicated in Fig. 4A, CmMRAP2 had no apparent effect, either negative or positive, on the functional expression of the SacMC<sub>5</sub> receptor.

The apparent absence of an interaction between the SacMC<sub>5</sub> receptor and CmMRAP2 would suggest that the tendency for the MC<sub>5</sub> receptor orthologs and the MRAPs to form a multimeric complex emerged after the divergence of the ancestral cartilaginous fish and the ancestral bony fish. However, the unexpected observation that mouse mMRAP1 and zebrafish zfMRAP1 had an effect on the functional expression of the SacMC<sub>5</sub> receptor (Fig. 4B) would indicate that the SacMC<sub>5</sub> receptor has the capability to interact with an MRAP1. Both mMRAP1 and zfMRAP1 increased the sensitivity of the receptor for ACTH. Since it is assumed that the dogfish genome lacks an *mrp1* gene, the effects observed in Fig. 4B are pharmacological, and the apparent increase in sensitivity may have been due to the MRAP1s interfering with the internalization of ligand-bound MC<sub>5</sub> receptor. Yet these observations may point to an ex-adaptation in the primary sequence of the SacMC<sub>5</sub> receptor that may be fundamental to understanding the origin of this melanocortin receptor paralogs. Alternatively, these observations may be the result of functional convergence.

## 5. Conclusions

It is perplexing that only three melanocortin receptor paralogs have been detected in the two elasmobranchs (*S. acanthias* and *Heterodontus francisci* Baron et al., 2009; Klovins et al., 2004b; Ringholm et al., 2003) that have been studied. It would appear that melanocortin receptor gene lost may have occurred during the radiation of the elasmobranchs since theoretically at least four melanocortin receptor paralogs should have been present in the genome of the ancestral cartilaginous fishes (Baron et al., 2009), and it is quite possible that all five melanocortin receptor paralogs were present in the genome of the ancestral cartilaginous fishes (Vastermark and Schiöth, 2011). Earlier studies on elasmobranchs indicated that injection of ACTH results in the release of a glucocorticoid from the head kidney (deRoos and deRoos, 1992). In addition, MC<sub>5</sub> receptor

expression has been detected in extracts of the interrenal tissue (Baron et al., 2009). Hence it is possible that the HPI axis in elasmobranchs can utilize the MC<sub>5</sub> receptor on the interrenal cells to induce glucocorticoid production. Given the ligands selectivity of the SacMC<sub>5</sub> receptor does this mean that products from either the anterior pituitary or the intermediate pituitary can induce the release of glucocorticoids, and if so what mechanisms determine which lobe of the pituitary will be used? The use of receptor expression in heterologous cells can provide useful information on the ligand selectivity of a receptor, however, to resolve questions related to the physiological role of the SacMC<sub>5</sub> receptor in the dogfish, in vivo studies are required.

## Acknowledgments

This research was supported by NSF grant IOB 0516958 (RMD). We wish to thank Nancy Lorenzon for her assistance with the immunocytochemical analysis.

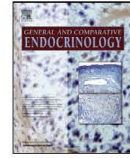
## References

- Agulleiro, M.J., Roy, S., Sanchez, E., Puchol, P., Gallo-Payet, N., Cerda-Reverter, J.M., 2010. Role of melanocortin receptor accessory proteins in the function of zebrafish melanocortin receptor type 2. *Mol. Cell. Endocrinol.* 320, 145–152.
- Amemiya, Y., Takahashi, A., Meguro, H., Kawachi, H., 1999. A newly characterized melanotropin in proopiomelanocortin in pituitaries of an elasmobranch, *Squalus acanthias*. *Gen. Comp. Endocrinol.* 114, 387–395.
- Baron, A., Veo, K., Angleson, J., Doros, R.M., 2009. Modeling the evolution of the MC2R and MC5R genes: studies on the cartilaginous fish *Heterodontus francisci*. *Gen. Comp. Endocrinol.* 161, 13–19.
- Chan, L.F., Webb, T.R., Chung, T., Meimaridou, E., Cooray, S., Guasti, L., Chapple, J.P., Egerova, M., Elphick, M.R., Cheetham, M.E., Metherell, L.A., Clark, A.J., 2009. MRAP and MRAP2 are bidirectional regulators of the melanocortin receptor family. *Proc. Natl. Acad. Sci. U. S. A.* 106, 6146–6151.
- Chepurny, O.G., Holz, G.G., 2007. A novel cyclic adenosine monophosphate responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein coupled receptor drug discovery efforts. *J. Biomol. Screen.* 12, 740–746.
- Cone, R.D., 2006. Studies on the physiological functions of the melanocortin system. *Endocr. Rev.* 27, 736–749.
- deRoos, R., deRoos, C.C., 1992. Effects of mammalian ACTH on potential fuels and glucogenic substrates in the plasma of the spiny dogfish shark (*Squalus acanthias*). *Gen. Comp. Endocrinol.* 87, 149–158.
- Doros, R.M., Rubin, D.A., Quinn, T.W., 1996. Mini-review: is it possible to construct phylogenetic trees using polypeptide hormone sequences. *Gen. Comp. Endocrinol.* 103, 1–12.
- Haitina, T., Takahashi, A., Holmen, L., Enberg, J., Schiöth, H.B., 2007a. Further evidence for ancient role of ACTH peptides at melanocortin (MC) receptors: pharmacology of dogfish and lamprey peptides at dogfish MC receptors. *Peptides* 28, 798–805.
- Haitina, T., Klovins, J., Takahashi, A., Löwgren, M., Ringholm, A., Enberg, J., Kawachi, H., Larson, E.T., Fredriksson, R., Schiöth, H.B., 2007b. Functional characterization of two melanocortin (MC) receptors in lamprey showing orthology to the MC1 and MC4 receptor subtypes. *BMC Evol. Biol.* 7, 101–115.
- Hinkle, P.M., Sebag, J.A., 2009. Structure and function of the melanocortin 2 receptor accessory protein. *Mol. Cell. Endocrinol.* 300, 25–31.
- Holland, P.W., Garcia-Fernandez, J., Williams, N.A., Sidow, A., 1994. Gene duplications and the origins of vertebrate development. *Development* 125–133 (Supplement).
- Klovins, J., Haitina, T., Ringholm, A., Löwgren, M., Fridmanis, D., Slaidina, M., Stier, S., Schiöth, H.B., 2004a. Cloning of two melanocortin (MC) receptors in spiny dogfish. *Eur. J. Biochem.* 271, 4320–4331.
- Klovins, J., Haitina, T., Fridmanis, D., Kilianova, Z., Kapa, I., Fredriksson, R., Gallo Payet, N., Schiöth, H.B., 2004b. The melanocortin system in *Fugu*: determination of POMC/AGRP/MCR gene repertoire and synteny, as well as pharmacology and anatomical distribution of the MCRs. *Mol. Biol. Evol.* 21, 563–579.
- Ling, M.K., Hotta, E., Kilianova, Z., Haitina, T., Ringholm, A., Johansson, L., Gallo-Payet, N., Takeuchi, S., Schiöth, H.B., 2004. The melanocortin receptor subtypes in chicken have high preference to ACTH-derived peptides. *British J. Pharmacology* 143, 626–637.
- Lundin, L.G., 1993. Evolution of the vertebrate genome as reflected in paralogous chromosomal regions in man and the house mouse. *Genomics* 16, 1–19.
- Metherell, L.A., Chapple, J.P., Cooray, S., David, A., Becker, C., Ruschendorf, F., Naville, D., Begeot, M., Khoo, B., Nurnberg, P., Huebner, A., Cheetham, M.E., Clark, A.J., 2005. Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. *Nat. Genet.* 37, 166–170.
- Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Cone, R.D., 1992. The cloning of a family of genes that encode the melanocortin receptors. *Science* 257, 543–546.
- Ohno, S., Wolf, U., Atkins, N.B., 1968. Evolution from fish to mammals by gene duplication. *Heredity* 59, 169–187.



- Pogozheva, I.D., Chai, B.-X., Lomize, A.L., Fong, T.M., Weinberg, D.H., Nargund, R.P., Mulholland, M.W., Grantz, I., Mosberg, H.I., 2005. Interactions of human melanocortin 4 receptors with nonpeptide and peptide agonists. *Biochemistry* 44, 11329–11341.
- Ringholm, A., Fredriksson, R., Poliakova, N., Yan, Y., Postlethwait, J.H., Larhammar, D., Schiöth, H.B., 2002. One melanocortin 4 and two melanocortin 5 receptors from zebrafish show remarkable conservation in structure and pharmacology. *J. Neurochem.* 82, 6–18.
- Ringholm, A., Klovins, J., Fredriksson, R., Poliakova, N., Larson, E.T., Kukkonen, J.P., Larhammar, D., Schiöth, H.B., 2003. Presence of melanocortin (MC4) receptor in spiny dogfish suggests an ancient vertebrate origin of central melanocortin system. *Eur. J. Biochem.* 270, 213–221.
- Roy, S., Rachel, M., Gallo-Payet, N., 2007. Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms  $\alpha$  and  $\beta$  in isogenic human embryonic kidney 293 cells. *Mol. Endocrinol.* 21, 1656–1669.
- Schiöth, H.B., Hältia, T., Ling, M.K., Ringholm, A., Fredriksson, R., Cerda-Reverter, J.M., Klovins, J., 2005. Evolutionary conservation of the structural pharmacological and genomic characteristics of the melanocortin receptor subtypes. *Peptides* 26, 1886–1900.
- Sebag, J.A., Hinkle, P.M., 2007. Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. *Proc. Natl. Acad. Sci. U. S. A.* 104, 20244–20249.
- Sebag, J.A., Hinkle, P.M., 2009a. Regions of melanocortin 2 (MC2) receptor accessory protein necessary for dual topology and MC2 receptor trafficking and signaling. *J. Biol. Chem.* 284 (2009), 610–618.
- Sebag, J.A., Hinkle, P.M., 2009b. Opposite effects of the melanocortin 2 (MC2) receptor accessory protein MRAP on MC2 and MC5 receptor dimerization and trafficking. *J. Biol. Chem.* 284, 22641–22648.
- Tan, C.M., Brady, A.E., Nickols, H.H., Wang, Q., Limbird, L.E., 2003. Membrane trafficking of G-protein-coupled receptors. *Ann. Rev. Pharmacol. Toxicol.* 44, 559–609.
- van der Kraan, M., Adan, R.A.H., Entwistle, M.L., Gispen, W.H., Burbach, J.P.H., Tatro, J.B., 1998. Expression of melanocortin-5-receptor in secretory epithelia supports a functional role in exocrine and endocrine glands. *Endocrinology* 139, 2348–2355.
- Vastermark, A., Schiöth, H., 2011. The early origin of melanocortin receptors, agouti-related peptide, agouti signaling peptide, and melanocortin receptor-accessory proteins, with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus. *Eur. J. Pharmacol.* doi:10.1016/j.ejphar.2010.10.106.
- Veo, K., Reinick, C., Liang, L., Moser, E., Angleson, J., Dore, R.M., 2011. Mini review: observations on the ligand selectivity of the melanocortin 2 receptor. *Gen. Comp. Endocrinol.* 172, 96–106.
- Webb, T.R., Clark, A.J., 2010. Minireview: the melanocortin 2 receptor accessory proteins. *Mol. Endocrinol.* 24, 1–10.
- Webb, T.R., Chan, T.K., Cooray, S.N., Cheetham, M.E., Chapple, J.P., Clark, A.J., 2009. Distinct melanocortin 2 receptor accessory protein domains are required for melanocortin 2 receptor interaction and promotion of receptor trafficking. *Endocrinology* 150, 720–726.

## **APPENDIX 5**



## Review

## Using the human melanocortin-2 receptor as a model for analyzing hormone/receptor interactions between a mammalian MC2 receptor and ACTH(1–24)

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## ARTICLE INFO

Article history:  
Available online 29 November 2012

Keywords:  
Human melanocortin-2 receptor  
HPA  
ACTH

## ABSTRACT

When considering the interactions between the melanocortin peptides (i.e., ACTH,  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH) and the melanocortin receptors (i.e., MC1R, MC2R, MC3R, MC4R, MC5R), it appears that the structure/function relationship between ACTH and MC2R is the most complicated. Human ACTH(1–24) and the human melanocortin-2 receptor provide a useful model system for understanding how ACTH emerged as the sole ligand for the melanocortin-2 receptor of bony vertebrates. This review will discuss how studies utilizing analogs of hACTH(1–24) have revealed two critical amino acid motifs in this ligand (HFRW and KKRRP) which are required for activation of the melanocortin-2 receptor. In addition, observations on the unique activation features of the melanocortin-2 receptor, as revealed from studies on Familial Glucocorticoid Deficiency, will be considered. Finally, the evolutionary implications of the relationship between MC2R and MRAP1 will be discussed.

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## 1. Introduction

ACTH (adrenocorticotropin) was one of the first pituitary hormones to be characterized [26], and although this 39-amino acid polypeptide is relatively small, the biochemical nature of this hormone is more complex than the other melanocortin peptides (i.e.,  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH). Studies conducted over 30 years ago established that all of the melanocortin-related peptides are derived from the polypeptide hormone precursor, POMC (proopiomelanocortin) [31]. In addition, it was found that melanocortins are synthesized in the corticotrophic cells of the pars distalis, and the melanotrophic cells of the pars intermedia [2]. Finally, in these pituitary cells types, differential posttranslational processing of POMC occurs to yield ACTH as the major melanocortin end-product of the corticotropes, and the MSHs as the major melanocortin end-products of the melanotropes [17]. In terms of function, studies on mammals established over 40 years ago that ACTH is the sole ligand for the “ACTH” receptor present on adrenal cortex cells [38]. A later study would reveal that the “ACTH” receptor is in fact the melanocortin-2 receptor (MC2R) [30]; a G-protein coupled receptor (GPCR) in the rhodopsin family [10]. Subsequent studies have discovered that the melanocortin-2 receptor has functional activation features which are distinct from the other melanocortin receptors (e.g., MC1R, MC3R, MC4R, MC5R) [23,42]. Hence, it

appears that the co-evolution of ACTH and MC2R has been shaped by selection pressures which have led to the one-on-one relationship between the ligand and the receptor that is observed in the hypothalamus/pituitary/adrenal axis of mammals. This review will use studies on mammalian melanocortin-2 receptors (principally human MC2R) to evaluate the structure/function features of ACTH, and to recapitulate the unique functional expression features of mammalian MC2Rs. In addition, a review of the mutations that have been detected in the human *mc2r* gene will underscore the value of using the human melanocortin-2 receptor as a model for comparative studies on orthologs of this receptor in other gnathostomes.

## 2. Structure/function analyses of ACTH(1–24)

As noted in Section 1, ACTH is a 39 amino acid polypeptide in most species of vertebrates [14]. However, earlier studies reviewed by Schwyzler [38] indicated that the functional domain required for activating the adrenal “ACTH” receptor was located within the first 24 residues of this hormone. Perhaps it is not a surprise that this region of ACTH is highly conserved among the gnathostomes [14]. For example as shown in Table 1 when the primary amino sequence of human ACTH(1–24) and dogfish (*Squalus acanthias*) ACTH(1–24) are compared, the sequence identity is 88%. As summarized by Schwyzler [38], analog studies using mammalian ACTH(1–24) have uncovered two functional motifs within ACTH; the HFRW motif and the KKRR motif. Utilizing alanine substitution

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**Table 1**  
A comparison of ACTH sequences.

HsACTH (1–39)	<u>S</u> YSME <u>HFRW</u> GKP <u>VGR</u> KRRP <u>IKVY</u> NGAEDESAEAFPLEP
XtACTH (1–39)	<u>S</u> YSME <u>HFRW</u> GKP <u>VGR</u> KRRP <u>IKVY</u> NGVEEESAENYPMEL
OmACTH (1–39)	<u>S</u> YSME <u>HFRW</u> GKP <u>VGR</u> KRRP <u>IKVY</u> NGVEEESSEAFPEM
SaACTH (1–39)	<u>S</u> YSME <u>HFRW</u> GKP <u>VGR</u> KRRP <u>IKVY</u> NSFEDESVENMGPEL
	[----- ACTH (1–24) -----]

The HFRW binding motif is shaded in gray. The K/RKRRP proposed binding site is underlined. Positions where substitutions have occurred are shaded in black. Hs (human ACTH: Accession No. CAG46625.1), Xt (*Xenopus tropicalis*: Accession No. NP 0011318.1), Om (*Onchorhynchus mykiss*: Accession No. WP 001118190), Sa (*Squalus acanthias*: Accession No. P01197).

	N-terminal		
1R	M---VQGSQRRLLGSLNST---PTAIPQLGLAANQT-GARCLEVVISDGL	50	
2R	MK-----HIINSYENINNT-----ARNNS-----DCPRVVLPEEI		
3R	MNAS-CCLPSVQPTLPNGS-----EHLQAPFFSNQSSAFCEQVFIKPEV		
4R	MVNSTHRGMHTSLHLNRRSSYRLHNSASESLGKGYSDGGCYEQLFVSPEV		
5R	MNSS--FHLHFLDLNLNAT---EGNLSGPNVKNKS--SPCEDMGIAVEV		
	-----TM1-----] IC1 [-----TM2-----		
1R	FLSLGLVSLVENALVVATIAKNRNLHSPMYCFICCLALSDLVSGSNVLE	100	
2R	FFTISIVGVLENLIVLLAVFKNKNLQAPMYFFICSLAISDMIGSLYKILE		
3R	FLSLGIVSLENILVILAVVRNGLHSPMYFFLCSLAVADMLVSVSNALE		
4R	FVTLGVISLLENILVIVAIKKNLHSPMYFFICSLAVADMLVSVNGSE		
5R	FLTGLVISLLENILVIGAIKKNLHSPMYFFVCSLAVADMLVSMSSAWE		
	---] EC1 [-----TM3-----]		
1R	TAVILLEAGALVARAAVLQQLDNVIDVITCSSMLSSLCFLGATAVDRIY	150	
2R	NLIILRNMGYLKPRGSFETTADDIIDLFLVLSLGSIFSLSVIAADRIY		
3R	TIMIAIVHSDYLTFFEDQFIQHMNDIFDSMICISLVASICNLLAIAVDRIY		
4R	TIVITLLNSTD-TDAQSFTVNIDNVIDSVICSSLLASICSLIAVDRIY		
5R	TITTYLLNNKHVIADAFVRHIDNVFDSMICISVVASMCSSLIAIAVDRIY		
	IC2 [-----TM4-----] EC2 [---		
1R	SIFYALRYHSIVTLPRARRAVAAIWVASVVFSTLFIAFYDHVAVLLCLVV	200	
2R	TIFHALRYHSIVTMRRTVVVLTVIWFCTGTGITMVIFSHHVPTVITETS		
3R	TIFYALRYHSIMTVRKALTLIVAIWVCCGVCVGVFIVYSESKMIVICLIT		
4R	TIFYALQYHNIMTVKRVGIIISCIWAACVTSGILFIIYSDSSAVIICLIT		
5R	TIFYALRYHHIMTARRSGAIIAGIWAFTGCGIVFIFYSESTYVILCLIS		
	-----TM5-----] IC3 [-----		
1R	FFLAMLVLMVAVLYVHMLARACQHAQGIARLH-KRQRPVHQGFGLKGAFTL	250	
2R	LFPLMLVFILCLYVHMFLLARSHTRKISTLP-----RANMKGAITL		
3R	MEFAMLLMGLTYVHMFLLARLHVKRIALPPADGVAPQGHSCMKGAVTI		
4R	MEFTMLALMASLYVHMFLLARLHIKRIAVLP--GTGAIRQGANMKGAITL		
5R	MEFAMFLLVSLYIHMFLARLHVKRIALP--GASSARQRTSMQGAFTV		
	-----TM6-----] EC3 [-----TM7-----		
1R	TILLGIFFLCWGPFFLHLTLIVLCPEHPTCGGIFKNFNLFALIIICNAII	300	
2R	TILLGVFIFCWAPFVLHVLMTFCPSNPYCACYMSLFPQVNGMLIMCNAVI		
3R	TILLGVFIFCWAPFVLHVLIICTPTNPYCICYTAHFNTYLVLMCNSVI		
4R	TILIGVVFVVCWAPFVLHVLIFYISCPNPYCVCFMSHFNLVILIMCNSII		
5R	TMLLGVFTVCWAPFVLHVLTLMLSCPQNYCSRFSHFNMVILIMCNSVM		
	-----] C-terminal		
1R	DPLIYAFHSQELRRITLKEVLTCSW	335	
2R	DPFIYAFRSPELRDAFKKMFCSRYW		
3R	DPLIYAFRSLELRNTFREILCGCNGMNLG		
4R	DPLIYALRSQELRKTTFKEIICCYPLGGLCDLSSRY		
5R	DPLIYAFRSQEMRKTTFKEIICRGFRIACSFPRRD		

**Fig. 1.** Comparison of the amino acid sequence of human melanocortin receptors. The amino acid sequences of human MC1R (Accession No. AAK58525), MC2R (Accession No. AA067714.1), MC3R (Accession No. AAB26088.1), MC4R (Accession No. AAB3341.1), and MC5R (Accession No. AAB60376.1) were aligned as described previously [15]. Potential N-linked glycosylation sites are underlined. Positions that are identical in at least four of the five sequences are shaded in gray. The primary sequence identity of the five paralogs was 42%. The (+) indicates positions in the human melanocortin-4 receptor that are found in the HFRW binding site of hMC4R [32]. Note the high level of primary sequence conservation at these sites in MC1R, MC3R, MC4R, and MC5R. Nine of the 10 sites are identical in these four paralogs, and six of the 10 sites are conserved in hMC2R.



analogs of hACTH(1–24), each of these motifs have been evaluated as described below.

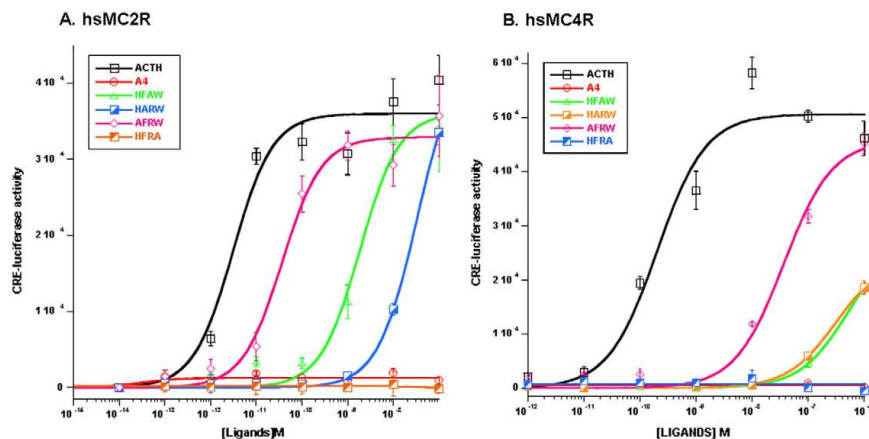
The HFRW motif is present in all of the melanocortins [31]. N-terminally truncated analogs of mammalian ACTH(1–24) in which this region of the polypeptide has been eliminated lack the ability to activate any melanocortin receptor [16,38]. Conversely, all melanocortin receptors have an HFRW binding site. The location of this site was determined by a modeling and site directed mutagenesis analysis of the human melanocortin-4 receptor [32]. As shown in Fig. 1, there are 10 critical amino acid positions distributed among TM2 (transmembrane domain 2), TM3, TM6, and TM7 of hMC4R which form this site. These amino acid positions are highly conserved in hMC1R, hMC3R, hMC4R, and hMC5R (Fig. 1). It is important to note that these four melanocortin receptors can be activated by  $\alpha$ -MSH as well as ACTH(1–24). Secondary structure analyses indicate that the HFRW motif in  $\alpha$ -MSH [N-acetyl-ACTH(1–13)amide] forms a reverse  $\beta$ -turn [22] that is then inserted into the corresponding binding site on the melanocortin receptor [32]; the result is activation.

Although the tertiary structure of hACTH(1–24) have not been determined, it is assumed that the HFRW motif in this ligand would also form a reverse  $\beta$ -turn as observed for  $\alpha$ -MSH. However, in the human MC2R sequence (Fig. 1) only six of the 10 residues in the proposed HFRW binding site are conserved as compared to hMC4R. Thus, the HFRW binding site on all melanocortin receptors may not be identical, or to look at this issue from a different perspective, perhaps some residues in the HFRW motif of hACTH(1–24) play a more or less prominent role in the activation of the human melanocortin-2 receptor as compared to the activation of the human melanocortin-4 receptor. Previous studies had used N-terminally truncated analogs of ACTH(1–24) [38] to address this question, but those analogs may have altered secondary structure. To minimize this issue, we tested alanine analogs of the HFRW motif in hACTH(1–24). As compared to hACTH(1–24) (Fig. 2A; Table 2), the AFRW analog with an alanine substitution at H<sup>6</sup> displayed a right shift on the activation curve. This resulted in an increase

of EC<sub>50</sub> value from  $4.2 \pm 1.3$  to  $36.0 \pm 11$  pM ( $P = 0.057$ ) as compared to stimulation by the wild type hACTH(1–24) (Table 2). Although the AFRW analog was ninefold less potent at activating the hMC2R, the  $P$  value (Table 2) was just below significance. By comparison, substitution at F<sup>7</sup> (analog HARW) and R<sup>8</sup> (analog HFAW) significantly reduced the potency of the ligands by nearly 8000-fold and 440-fold, respectively. The EC<sub>50</sub> values and  $P$  values for these analogs are shown in Table 2. Interestingly, the analog HFRA, in which W<sup>9</sup> was replaced by an alanine, failed to stimulate hMC2R even at the highest concentration ( $10^{-6}$  M) tested (Fig. 2A). The same observation was obtained when the entire HFRW motif were replaced by alanines (analog A4). These observations would indicate that tryptophan is the most important residue in the HFRW motif and is absolutely required for activation of the receptor.

When the same hACTH(1–24) analogs were used to stimulate the human melanocortin-4 receptor, a slightly different profile was observed (Fig. 2B). For this receptor no activation was observed when the receptor was incubated with either analog A4 or analog HFRA (Table 2). In addition, the EC<sub>50</sub> value of AFRW increased by about 100-fold as compared to hACTH(1–24) (Table 2), and this shift was statistically significant ( $P < 0.05$ ). Furthermore, stimulation with the HARW analog and HFAW analog was extremely weak (15,000 and 26,000 increase in EC<sub>50</sub>, respectively) as compared to stimulation by hACTH(1–24) (Table 2). As shown in Fig. 2B, alanine substitutions to the HFRW motif at F<sup>7</sup> and R<sup>8</sup> of ACTH(1–24) have a more pronounced negative effect on the activation of hMC4R as compared to hMC2R. Collectively these results point to 3-dimensional differences in the HFRW binding site of hMC2R as compared to hMC4R.

Given these observations we thought it would be reasonable to re-evaluate the KKRR motif in hACTH(1–24) (Table 1) by analyzing alanine-substitution analogs in this region of hACTH(1–24). The importance of this motif became apparent from studies on C-terminally truncated analogs of hACTH(1–24) [4,5,38]. Those analyses indicated that hACTH(1–16) was the shortest analog which could



**Fig. 2.** Activation of hMC2R and hMC4R by analogs in the HFRW motif of hACTH(1–24). (A) CHO cells were transfected with the following plasmids: human *mc2r* (2  $\mu$ g), mouse *mrp1* (2  $\mu$ g) and cAMP reporter plasmid *cre-luc* (2  $\mu$ g) [6] using a Cell Line Nucleofector Kit (Amaya, Inc.; www.lonza.com) as described in Liang et al. [27]. The cells were plated on a white, 96-well plate at a density of  $1 \times 10^5$  cells per well. Forty eight hours after transfection, cells were stimulated with the appropriate ligands in serum-free CHO media for four hours at 37 °C at concentrations ranging from  $10^{-6}$  to  $10^{-16}$  M. All assays were done in triplicate. After a four hour incubation period, 100  $\mu$ l of Bright-Glo luciferase assay reagent (Promega Inc., Madison, WI) was applied to each well and incubated at room temperature for 5 min. Luminescence was then measured with a Bio-Tek Synergy HT plate reader (Winooski, VT). The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: the hACTH(1–24), hACTH(1–24) A<sup>6</sup> [AFRW], hACTH(1–24) A<sup>7</sup> [HARW], hACTH(1–24) A<sup>8</sup> [HFAW], hACTH(1–24) A<sup>6</sup>A<sup>7</sup>A<sup>8</sup> [A4], and hACTH(1–24) A<sup>6</sup>A<sup>7</sup>A<sup>8</sup> [A4]. (B) CHO cells were transfected as described in (A) with the following plasmids: human *mc4r* (2  $\mu$ g) and *cre/luc* (2  $\mu$ g), and activation assays were performed as described in (A). The *hmc4r*-transfected cells were stimulated with the same ligands used in (A).  $N = 3$ .

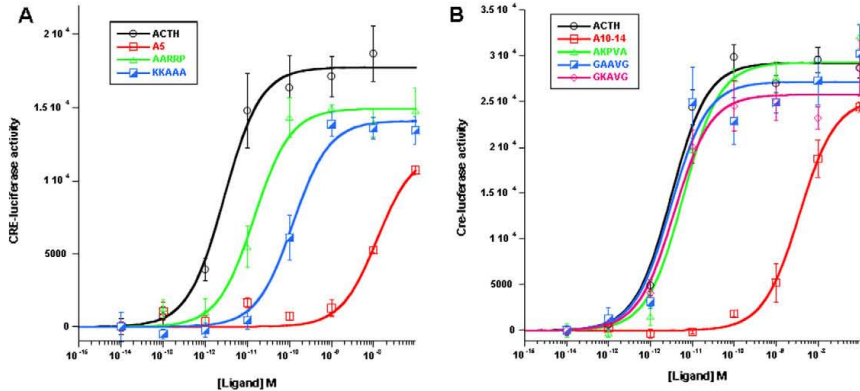
**Table 2**  
Summary of the ACTH(1–24) alanine substitution study.

Analogs	Amino acid sequences	hMC2R		hsMC4R	
		EC <sub>50</sub> ± SEM (pM)	P values	EC <sub>50</sub> ± SEM (nM)	P values
ACTH(1–24)	SYSMEHFRWGKPVGKKRRPVKVYP	4.2 ± 1.3		0.2 ± 0.85	
A4	SYSMEAAAAGKPVGKKRRPVKVYP	NA		NA	
AFRW	SYSMEAFRWGKPVGKKRRPVKVYP	36 ± 11	0.057	36 ± 8.9*	0.008
HARW	SYSMEHARWGKPVGKKRRPVKVYP	33505 ± 2559***	0.0001	3128 ± 424**	0.0009
HFAW	SYSMEHFAWGKPVGKKRRPVKVYP	1845 ± 467**	0.008	5284 ± 580***	0.0004
HFRA	SYSMEHFRAGKPVGKKRRPVKVYP	NA		NA	
A10–14	SYSMEHFRWAAAAGKRRPVKVYP	3608 ± 381***	0.0004		
AKPVA	SYSMEHFRWAKPVGKKRRPVKVYP	6.1 ± 1.7	0.2		
GAAVG	SYSMEHFRWGAAGKRRPVKVYP	3.1 ± 1.4	0.7		
GKAVG	SYSMEHFRWGAAGKRRPVKVYP	3.7 ± 1.1	0.6		
A5	SYSMEHFRWGKPVGAAAAVKVYP	12214 ± 4745*	0.03		
AARRP	SYSMEHFRWGKPVGAARRPVKVYP	14.4 ± 3.3*	0.02		
KKAAA	SYSMEHFRWGKPVGKAAAVKVYP	120 ± 24.5**	0.004		

EC<sub>50</sub> values are expressed as mean ± SEM (n = 3). Statistical differences were assessed by Student t-test. NA, No activation.  
\* P < 0.05.  
\*\* P < 0.005.  
\*\*\* P < 0.0005.

still induce a minimal biological response when incubated with rat adrenal cortex cells in culture [3] or in a mammalian OS3 adrenal cell line transfected with a *hmc2R* cDNA [5]. While the progressive loss of biological activity of the C-terminally truncated hACTH(1–24) analogs could be attributed to a conformational change in the analogs, the observation that the analog KPVGKKRRPVKVYP can act as a competitive inhibitor of ACTH(1–24) [41] provided evidence for a KKRR binding site in MC2R in addition to the HFRW binding site. A later study that utilized alanine substitutions in hACTH(1–24) underscored the importance of P<sup>19</sup> in this proposed binding site [12]. In Fig. 3A, we looked at the relative importance of the K<sup>15</sup>K<sup>16</sup> motif as compared to the R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> motif in the activation process. As indicated in Table 2, both analogs showed a statistically significant increase in EC<sub>50</sub> values as compared to hACTH(1–24). Stimulation with the AARRP analog resulted in a threefold increase in EC<sub>50</sub> relative to the positive control. Although this shift in EC<sub>50</sub> was statistically significant (Table 2), the fold shift appears to be marginal. However, stimulation with the KKAAA analog resulted in a 29-fold increase in the EC<sub>50</sub> value (Table 1). When

all the positions in this motif were replaced with alanines (A5 analog) there was a 3000-fold increase in the EC<sub>50</sub> relative to the positive control (Table 2, Fig. 3A). However, it was interesting that the A5 analog did not totally block activation of the hMC2R; whereas, substitution of alanines in the HFRW motif of the ligand (A4 analog; Fig. 2A) completely inhibited activation of the receptor. Perhaps positions 20–24 in ACTH(1–24) also play a role in the activation of the receptor. This possibility should be explored. Accepting the premise that there is an HFRW binding site as well as a KKRRP binding site on hMC2R, we next wished to address the question of the relative distance between these two binding sites. The operating hypothesis was that binding of hACTH(1–24) at the proposed KKRRP binding site would position the HFRW domain of hACTH(1–24) into its corresponding binding site on the receptor. A corollary to this hypothesis would be that the G<sup>10</sup>K<sup>11</sup>-P<sup>12</sup>V<sup>13</sup>G<sup>14</sup> motif would play a critical role in the positioning of the ligand in these two binding sites. To test this hypothesis, the activation activity of analogs with alanine substitutions in this region of hACTH(1–24) was tested (Fig. 3B). Analogs with either

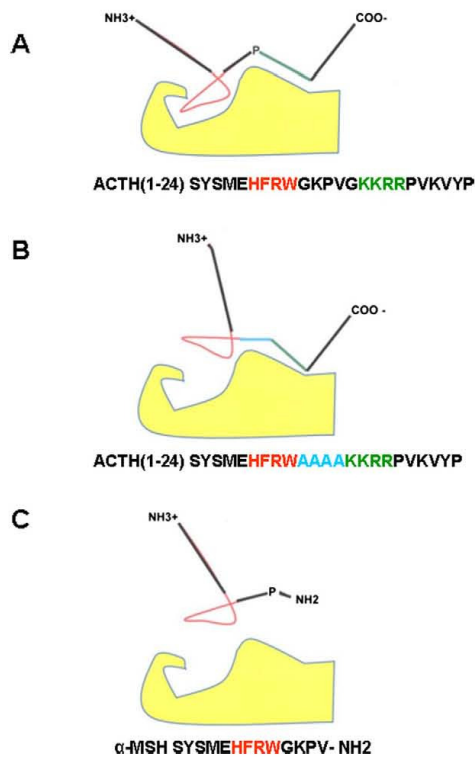


**Fig. 3.** Activation of hMC2R by analogs of hACTH(1–24) in the KKRRP motif and the GKPVG motif. (A) CHO cells were transfected with the following plasmids as described for Fig. 2A: *hmc2R*, mouse *mrp1*, and *cre/luc*. Activation assays were done as described for Fig. 2A. The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: the hACTH(1–24), hACTH(1–24) A<sup>15</sup>A<sup>16</sup> [AARRP], hACTH(1–24) A<sup>17</sup>A<sup>18</sup>A<sup>19</sup> [KKAAA], and hACTH(1–24) A<sup>15</sup>A<sup>16</sup>A<sup>17</sup>A<sup>18</sup>A<sup>19</sup> [A5]. N = 3. (B) CHO cells were transfected with the following plasmids as described for Fig. 2A: *hmc2R*, mouse *mrp1*, and *cre/luc*. Activation assays were done as described for Fig. 2A. The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: the hACTH(1–24), hACTH(1–24) A<sup>12</sup> [GKAVG], hACTH(1–24) A<sup>11</sup>A<sup>12</sup> [GAAVG], hACTH(1–24) A<sup>10</sup>A<sup>14</sup> [AKPVA], and hACTH(1–24) A<sup>10</sup>A<sup>11</sup>A<sup>12</sup>A<sup>13</sup>A<sup>14</sup>. N = 3.



single alanine or double alanine substitutions did not affect the activation curves of hMC2R (see EC<sub>50</sub> values in Table 2). However, when all amino acid positions in this motif were replaced with alanines (A10–14 analog), there was a significant reduction in potency resulting in a 900-fold right shift in the activation curve.

It would appear that a change in the secondary structure of the A(10–14) analog affected activation of the receptor. A model summarizing these observations is presented in Fig. 4. This model assumes that initial contact with the hMC2R occurs at the proposed KKRRP binding site. The model assumes that the secondary structure of the G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup> region of hACTH(1–24) properly positions the HFRW motif of the ligand into the HFRW binding site on the receptor (Fig. 4A). Altering the secondary structure of the GPKVG motif could miss orient the N-terminal of the ligand in the HFRW binding site on the receptor (Fig. 4B) or interferes with binding at the proposed KKRRP binding site on the receptor.



**Fig. 4.** Modeling the activation of hMC2R. (A) The operating assumption is that the human melanocortin-2 receptor has two distinct binding sites; a binding site for the HFRW motif in ACTH(1–24) that when occupied induces the activation of the G-Protein associated with the receptor, and the KKRRP binding site; the proposed first contact site on the receptor. When the KKRRP binding site is occupied by the ligand, the HFRW motif on the ligand is now properly positioned for interaction with the HFRW binding site. (B) In order for the ligand to occupy both binding sites, the secondary structure of the G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup> cannot be altered. In support of this hypothesis, when the G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup> motif in the ligand is replaced with alanines, either the N-terminal domain or the C-terminal domain of the ligand is out of position, and the result is a dramatic drop in activation potency of the ligand. (C) The conclusions drawn from (A) and (B) would explain why α-MSH [N-Ac-ACTH(1–13)amide] cannot activate hMC2R or act as a competitive inhibitor of MC2R [3]. α-MSH lacks the C-terminal KKRRP extension. Therefore, this ligand cannot bind to hMC2R at the KKRRP binding site. As a result, α-MSH cannot make contact with the HFRW binding site on the receptor.

Based on these assumptions, this model would explain the inability of α-MSH to activate MC2R (Fig. 4C), or even to act as a competitive inhibitor of ACTH(1–24) [4]. Since α-MSH lacks the KKRRP motif, this ligand cannot be properly oriented for insertion at the HFRW binding site on the melanocortin-2 receptor. However, it should be noted that either α-MSH or ACTH can enter the HFRW binding site in MC1R, MC3R, MC4R, or MC5R and activate these receptors [10]. Clearly, the linear sequence data provided in Fig. 1 does not reveal the 3-dimensional features which distinguish the HFRW binding site in MC2R from the corresponding binding site in MC1R, MC3R, MC4R or MC5R. It should be noted that ACTH stimulation of target cells not only results in an increase in intracellular cAMP, but also leads to an inward Ca<sup>2+</sup> flux [9,13,20,21,25]. The possibility that the ACTH analogs used in this study are affecting Ca<sup>2+</sup> influx was not tested.

### 3. Role of MRAP1 in the activation of the melanocortin-2 receptor

Among the actinopterygian and sarcopterygian gnathostomes perhaps the most enigmatic aspect of the ACTH/MC2R relationship has been the realization that this interaction requires the intercession of the accessory protein, MRAP1 (melanocortin-2 receptor accessory protein 1) [28]. It should be noted that the early structure/function studies utilizing analogs of ACTH(1–24) were done on explants of mammalian adrenal cortex tissue [38] or isolated adrenal cortex cells [3]. The characterization of the mouse melanocortin-2 receptor by Mountjoy et al. [30] was done in Cloudman S91 melanoma cells. However, attempts to functionally express mammalian *mc2r* cDNAs in mammalian cell lines such as HEK-293 cells, CHO cells, or COS cells were completely unsuccessful, even though the other melanocortin receptor paralogs (i.e., MC1, MC3R, MC4R, MC5R) could be functionally expressed in these cell lines [18,24,33,35]. It appeared that some cell types, such as adrenal cortex cells and adipose cells, had an additional factor which facilitated the functional expression of the melanocortin-2 receptor.

Support for this hypothesis came from studies on Familial Glucocorticoid Deficiency (FGD). FGD is a congenital syndrome in humans which results in a life-threatening underproduction of cortisol by adrenal cortex cells. For some patients the problem is an error in the biosynthetic pathway for making cortisol from cholesterol [29]. For other patients the cortisol deficiency is due to mutations in the melanocortin-2 receptor [8]. However, for a subset of patients the defect which resulted in FGD is not a mutation in the glucocorticoid biosynthetic pathway or the melanocortin-2 receptor, but rather a mutation in an accessory protein expressed in adrenal cortex cells (Type 2; [28]). The accessory protein is MRAP [28].

MRAPs are single chain polypeptides with a single membrane spanning domain [28]. Two MRAP paralogs have been characterized from the genomes of vertebrates [23,42], and are designated MRAP1 and MRAP2. Both paralogs have the distinctive feature of forming homodimers in which the two proteins are oriented in an antiparallel manner in the membrane of the endoplasmic reticulum [39,40]. Both the MRAP1 homodimer and the MRAP2 homodimer appear to independently form a complex with the melanocortin-2 receptor at the endoplasmic reticulum. While both the MRAP1/MC2R and the MRAP2/MC2R complexes will move to the plasma membrane, only the MRAP1/MC2R complex can efficiently bind ACTH and be activated [23,42]. The reason for the differences in the functions of mammalian MRAP1 and MRAP2 resides in the amino acid motif, LDYL/I [40]. This amino acid motif is present in the N-terminal of mammalian MRAP1s, but absent from the N-terminal of mammalian MRAP2s, and is responsible for inducing

the conformation change in MC2R which makes the receptor functionally active. Finally, a study on the co-expression of human MRAP1 and human MC2R in mammalian HEK-293 cells indicated that the MRAP1/MC2R complex is a hexameric oligomer consisting of two MRAP1 homodimers and one MC2R homodimer [11,37]. In Figs. 2, 3 and 6 the functional expression of human MC2R was done by also co-expressing mouse MRAP1 in the CHO cells. A previous study indicated that there was no evidence that MRAPs are expressed in the CHO cells used in this study [34].

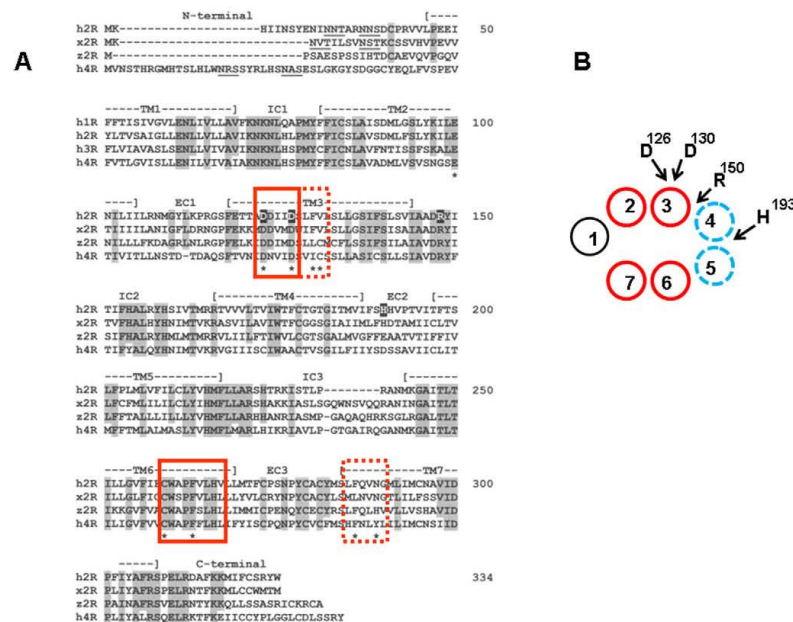
The significance of the interaction between ACTH, MC2R, and MRAP1 cannot be understated. As reviewed recently [23,42], when the melanocortin-2 receptor is expressed alone, the receptor is folded improperly. As a result, the misshaped receptor will be retained in the endoplasmic reticulum and eventually degraded. Hence, while Fig. 1 shows the amino acid positions in the linear sequence of human MC2R that most likely form the HFRW binding site, unless the melanocortin-2 receptor forms a complex with MRAP1, the receptor will not be in the appropriate 3-dimensional conformation required for activation. In addition, without MRAP present to serve as a trafficking chaperone, the MC2 receptor will be unable to leave the endoplasmic reticulum.

For humans the various types of Familial Glucocorticoid Deficiency can be treated by taking cortisol supplements. Could this syndrome occur in a non-mammalian vertebrate? The answer is, most likely yes. Recent studies indicate that the melanocortin-2 receptor for the zebrafish, *Danio rerio* [1] and the frog, *Xenopus tropicalis* [27] both require co-expression with an MRAP1 in order

to be functionally activated. Hence, if mutations occur in a non-mammalian bony vertebrate in either the *mc2r* gene which affect either trafficking or activation, or if mutations occur in the corresponding sites in the *mrp1* gene that affect either trafficking or activation of MC2R, the outcome would most likely be deleterious to the fitness of the organism.

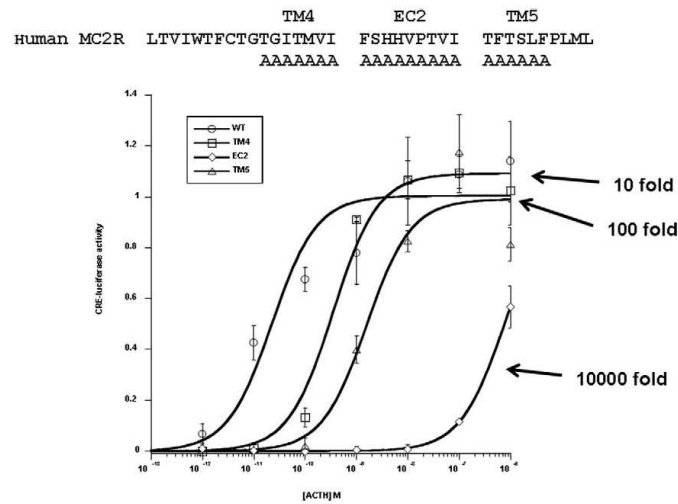
This conclusion is not surprising after comparing the linear sequences of human, zebrafish, and *X. tropicalis* melanocortin-2 receptors as shown in Fig. 5A. Note that all three orthologous MC2R sequences have identical sequences at positions 103, 126, 130, 260, 263, and 266 in the proposed HFRW binding site (red boxes), and variable sequences at positions 133, 134 (TM3), and 291, 294 (TM7) as compared to hMC4R.

In this regard, naturally occurring mutations which inhibit activation of human MC2R, resulting in FGD, have been detected at the conserved positions 126 and 130 in TM3 (Fig. 5A). Tracking other naturally occurring activation inhibiting mutations may point to other functional domains in the receptor that are conserved among the bony vertebrates [7,19]. For example, an activation inhibiting mutation has been detected at R<sup>150</sup> in the DRY motif. This motif is critical for proper interaction between the receptor and the G-Protein which leads to the activation of adenylyl cyclase [7]. An apparently perplexing activation inhibiting mutation has also been observed at H<sup>193</sup> in extracellular loop 2 (EC2) [7] of human MC2R. Extracellular Loop 2 is outside of the HFRW binding site. Assuming that the 3-dimensional structure of MC2R orients the seven transmembrane regions in a barrel conformation as shown in Fig. 5B,



**Fig. 5.** Alignment of human, frog, and zebrafish MC2Rs. (A) The amino acid sequence of human MC2R (Accession No. AA067714.1), *Xenopus tropicalis* MC2R (Accession No. XP\_002933963), and zebrafish (*Danio rerio*; Accession No. AA024743.1) were aligned as described in the legend for Fig. 1 and compared to the sequence of human MC4R (Accession No. AAB3341.1). Amino acid positions that are identical in all three MC2R sequences are shaded (note some of these positions are also identical in hMC4R). Among the three MC2R orthologs, 28% of the positions are identical. The amino acid positions that are found in the HFRW binding site of human MC4R are marked with a star (\*) [25]. A box with a solid red line indicates that the residues in the HFRW binding site are identical in both the MC2R sequences and the hMC4R sequence. A box with a dashed line indicates a region of the hMC4R HFRW binding site in which the residues are different from the corresponding positions in the MC2R sequences. An amino acid position shaded in black indicates a site where a mutation in the human MC2R sequence that blocks activation of the receptor, resulting in Familial Glucocorticoid Deficiency, has been reported [7]. (B) A schematic representation of the proposed orientation of transmembrane (TM) domains (colored circles) of hMC2R. The transmembrane domains that comprise the HFRW binding site are in red (solid circle). The transmembrane domains that may be in the proposed KRRP binding site are in blue (dashed circle). The relative positioning of naturally occurring mutants in human MC2R which have a negative effect on activation are shown.





**Fig. 6.** The effects of alanine mutations in TM4, EC2, and TM5 of hMC2R. Three multiple alanine mutants of human MC2R were synthesized by GenScript (Piscataway, NJ) and inserted into the pcDNA3.1 + expression vector: hMC2R TM4 mutant (A<sup>161</sup>–A<sup>167</sup>), hMC2R EC2 mutant (A<sup>168</sup>–A<sup>176</sup>), and hMC2R TM5 (A<sup>177</sup>–A<sup>183</sup>). Each mutant construct was separately transfected into CHO cells with the mouse *mrap1* plasmid and the *cre/luc* plasmid as described for Fig. 2A. After 48 h in culture, transfected cells were stimulated for 4 h with hACTH(1–24) at concentrations ranging from 10<sup>–12</sup> to 10<sup>–6</sup> M, and activation was determined as described for Fig. 2A. The positive control was to transfect CHO cells with the wild-type (wt) human *mc2r* plasmid, mouse *mrap1* plasmid, and *cre/luc* plasmid as described for Fig. 2A. *N* = 3.

then TM2, TM3, TM6, and TM7 which form the HFRW binding site of MC2R [7], would be in close proximity. In this model EC2 could be positioned in the proposed KKRRP binding site flanked by TM4 and TM5.

To test this hypothesis alanine substitution mutants were made in TM4, EC2, and TM5, respectively, and the ability of hACTH(1–24) to activate these hMC2R mutants was tested (Fig. 6). Receptor activation by hACTH(1–24) for all three mutants was significantly reduced, but to different degrees, as compared to the wild type hMC2R. The EC2 mutant displayed a 10,000-fold right shift, suggesting an essential role of extracellular loop 2 in the binding of ACTH to the receptor. Meanwhile, TM4 and TM5 mutants showed a 10- and 100-fold increase in EC<sub>50</sub> values, respectively. While the outcome of this experiment is intriguing, the actual roles of these three regions in the activation of hMC2R will need to be tested by analyzing single alanine mutations in these regions of the receptor.

#### 4. Conclusions

When considering the factors which distinguish the human melanocortin-2 receptor from the other human melanocortin receptors (i.e., MC1R, MC3R, MC4R, MC5R), three features stand-out. First, while the primary sequence of the human melanocortin-2 receptor indicates that many of the amino acid positions found in the HFRW binding site of the human melanocortin-4 receptor are present (Fig. 1), these two binding sites do not operate in an identical manner. As indicated in the ligand activation studies, analysis of the HFRW motif of hACTH(1–24) indicated the following order of importance for activation of hMC2R: W<sup>9</sup> > R<sup>8</sup> > F<sup>7</sup> > H<sup>6</sup>. However, for hMC4R the order of importance of the residues in the HFRW motif of ACTH(1–24) is W<sup>9</sup> >> F<sup>7</sup> > R<sup>8</sup> > H<sup>6</sup> (Fig. 2). These observations would suggest that the 3-dimensional structure of the “HFRW” binding sites in hMC2R and hMC4R, may be similar, but not identical. While it has already been shown that α-MSH cannot activate the zebrafish melanocortin-2 receptor [1] or the *X. tropicalis* melanocortin-2 receptor [27], it is

not known whether these non-mammalian MC2Rs react to the HFRW analogs of hACTH(1–24) in the same manner as the human melanocortin-2 receptor.

Another unique feature of the human melanocortin-2 receptor is that activation by ACTH(1–24) is dependent on the KKRRP motif in this ligand. The operating model (Fig. 4) is that the ligand first makes contact with the receptor at a proposed KKRRP binding site and then activation is possible when the HFRW motif of the ligand is inserted into the corresponding binding site on the receptor. Within this region of ACTH(1–24), the RRP motif is more critical than the KK motif. In several non-mammalian vertebrates the sequence at position 16–19 in ACTH(1–24) is RKRRP (Table 1) [14]. The prediction would be that the substitution of an arginine residue for a lysine residue at position 16 of ACTH(1–24) will not interfere with the activation of non-mammalian MC2Rs.

Finally, during the initial synthesis of the human melanocortin-2 receptor at the rough endoplasmic reticulum the receptor is in an inactive conformation that cannot move independently to the plasma membrane [40]. However, if MRAP1 or MRAP2 is present at the rough endoplasmic reticulum, either of these accessory proteins can rescue the melanocortin-2 receptor, and facilitate the trafficking of the receptor to the plasma membrane [23,36,37,42]. However, of the two MRAP paralogs, only MRAP1 is capable of facilitating both the trafficking of hMC2R to the plasma membrane and the functional activation of the receptor at the plasma membrane. Identical observations have been made for the functional activation of zebrafish MC2R [1] and *X. tropicalis* MC2R [27]. Although the sample set is limited to three species (i.e., human, frog, and teleost fish), the phylogenetic positions of these organisms would suggest that in the ancestral bony fishes a functionally mutated melanocortin-2 receptor was rescued by an MRAP that was co-synthesized in cells expressing the ancestral *mc2r* gene. The preceding scenario begs the question of whether there is an MRAP1 independent melanocortin-2 ortholog in any extant gnathostome. Studies on melanocortin-2 orthologs in non-teleost bony fishes or in cartilaginous fishes will resolve this question.

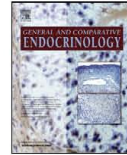
## Acknowledgment

This research was supported by NSF Grant IOB 0516958 (R.M.D.).

## References

- [1] M.J. Agulleiro, S. Roy, E. Sanchez, S. Puchol, N. Gallo-Payet, J.M. Cerda-Reverter, Role of melanocortin receptor accessory proteins in the function of zebrafish melanocortin receptor type 2, *Mol. Cell. Endocrinol.* 320 (2010) 145–152.
- [2] B. Baker, The evolution of ACTH, MSH, and LPH-structure, function, and development, *Horm. Evol.* 2 (1980) 643–722.
- [3] D.I. Buckley, J. Ramachandran, Characterization of corticotropin receptors on adrenocortical cells, *Proc. Natl. Acad. Sci. U S A* 78 (1981) 7431–7435.
- [4] D.I. Buckley, D. Yamashiro, J. Ramachandran, Synthesis of a corticotropin analogue that retains full biological activity after iodination, *Endocrinology* 109 (1981) 5–9.
- [5] M. Chen, C.J. Aprahamian, R.A. Kesterson, C.M. Harmon, Y. Yang, Molecular identification of the human melanocortin-2 receptor responsible for ligand binding and signaling, *Biochemistry* 46 (2007) 11389–11397.
- [6] O.G. Chepur, G.G. Holz, A novel cyclic adenosine monophosphate responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein coupled receptor drug discovery efforts, *J. Biomol. Screen.* 12 (2007) 740–746.
- [7] T.T. Chung, T.R. Webb, L.F. Chan, S.N. Cooray, L.A. Metherell, P.J. King, J.P. Chapple, A.J.L. Clark, The majority of ACTH receptor (MC2R) mutations found in Familial Glucocorticoid Deficiency type 1 lead to defective trafficking of the receptor to the cell surface, *J. Clin. Endocrinol. Metab.* 93 (2008) 4948–4954.
- [8] A.J.L. Clark, L. McLoughlin, A. Grossman, Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor, *Lancet* 341 (1993) 461–462.
- [9] A.J.L. Clark, F. Cammas, The ACTH receptor, *Baillieres Clin. Endocrinol. Metab.* 10 (1996) 29–47.
- [10] R.D. Cone, Studies on the physiological functions of the melanocortin system, *Endocr. Rev.* 27 (2006) 736–749.
- [11] S.N. Cooray, T.-I. Chung, K. Mazhar, L. Szidonya, A.J.L. Clark, Bioluminescence resonance energy transfer reveals the adrenocorticotropin (ACTH)-induced conformational change of the activated ACTH receptor complex in living cells, *Endocrinology* 152 (2011) 495–502.
- [12] J.L. Costa, S. Bui, P. Reed, R.M. Dore, U. Hochgeschwender, M.B. Brennan, Mutational analysis of evolutionarily conserved ACTH residues, *Gen. Comp. Endocrinol.* 136 (2004) 12–16.
- [13] M. Cote, G. Guillon, M. Payet, N. Gallo-Payet, Expression and regulation of adenylyl cyclase isoforms in the human adrenal gland, *J. Clin. Endocrinol. Metab.* 86 (2001) 4495–4503.
- [14] R.M. Dore, S. Lecaude, Trends in the evolution of the proopiomelanocortin gene, *Gen. Comp. Endocrinol.* 142 (2005) 81–93.
- [15] R.M. Dore, D.A. Rubin, T.W. Quinn, Mini-review: is it possible to construct phylogenetic trees using polypeptide hormone sequences, *Gen. Comp. Endocrinol.* 103 (1996) 1–12.
- [16] A. Eberle, R. Schwyzler, Hormone-receptor interactions. Demonstration of the two message sequences (active sites) in  $\alpha$ -melanotropin, *Helv. Chim. Acta* 58 (1975) 1528–1535.
- [17] B.A. Eipper, R.E. Mains, Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides, *Endocr. Rev.* 1 (1980) 1–27.
- [18] F.L. Forti, M.H.S. Dias, H.A. Armelin, ACTH receptor: ectopic expression, activity and signaling, *Mol. Cell. Biochem.* 293 (2006) 147–160.
- [19] D. Fridmanis, R. Petrovska, I. Kalnina, M. Slaidina, R. Peculis, H.B. Schioth, J. Klovins, Identification of domains responsible for specific membrane transport and ligand specificity of the ACTH receptor (MC2R), *Mol. Cell. Endocrinol.* 321 (2010) 175–183.
- [20] N. Gallo-Payet, E. Grazzini, M. Cote, L. Bilodeau, A. Chorvatova, M.D. Payet, L. Chouinard, G. Guillon, Role of calcium in the mechanism of action of ACTH in human adrenocortical cells, *J. Clin. Invest.* 98 (1996) 460–466.
- [21] N. Gallo-Payet, M.D. Payet, Mechanism of action of ACTH: beyond cAMP, *Microsc. Res. Tech.* 61 (2003) 275–287.
- [22] C. Haskell-Luevano, T.K. Sawyer, S. Trumpp-Kallmeyer, J.A. Bikker, C. Humblet, I. Gantz, V.J. Hruby, Three-dimensional molecular model of the hMC1R receptor melanocortin receptor: complexes with melanotropin peptide agonists, *Drug Des. Discov.* 14 (1996) 197–211.
- [23] P.M. Hinkle, J.A. Sebag, Structure and function of the melanocortin 2 receptor accessory protein, *Mol. Cell. Endocrinol.* 300 (2009) 25–31.
- [24] Z. Killanova, N. Basora, P. Killan, M.D. Payet, N. Gallo-Payet, Human melanocortin receptor 2 expression and functionality: effects of protein kinase A and protein kinase C on desensitization and internalization, *Endocrinology* 147 (2006) 2325–2337.
- [25] I. Kojima, K. Kojima, H. Rasmussen, Role of calcium and cAMP in the action of adrenocorticotropin on aldosterone secretion, *J. Biol. Chem.* 260 (1985) 4248–4256.
- [26] C.H. Li, L.I. Geschwind, R.D. Cole, L.D., Raake, J.I. Harris, J.S. Dixon, Amino-acid sequence of alpha-corticotrophin, *Nature* 176 (1955) 687–689.
- [27] L. Liang, J.A. Sebag, L. Egelston, M.N. Serasinghe, K. Vee, C. Reinick, J. Angleson, P.M. Hinkle, R.M. Dore, Functional expression of frog and rainbow trout melanocortin 2 receptors using heterologous MRAP1s, *Gen. Comp. Endocrinol.* 174 (2011) 5–14.
- [28] L.A. Metherell, J.P. Chapple, S. Cooray, A. David, C. Becker, F. Ruschendorf, D. Naville, M. Begeot, B. Khoo, P. Nurnberg, A. Huebner, M.E. Cheethan, A.J. Clark, Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2, *Nat. Genet.* 37 (2005) 166–170.
- [29] L.A. Metherell, D. Naville, G. Halaby, M. Begeot, A. Huebner, G. Nurnberg, P. Nurnberg, J. Green, J.W. Tomlinson, N.P. Krone, L. Lin, M. Racine, D.M. Berney, J.C. Achermann, W. Arlt, A.J. Clark, Non-classic lipid congenital adrenal hyperplasia masquerading as familial glucocorticoid deficiency, *J. Clin. Endocrinol. Metab.* 94 (2009) 3865–3871.
- [30] K.G. Mountjoy, L.S. Robbins, M.T. Mortrud, R.D. Cone, The cloning of a family of genes that encode the melanocortin receptors, *Science* 257 (1992) 1248–1251.
- [31] S. Nakanishi, A. Inoue, T. Kita, M. Nakamura, A.C.Y. Chung, S.N. Cohen, S. Numa, Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor, *Nature* 278 (1979) 423–427.
- [32] I.D. Pogozheva, B.-X. Chai, A.L. Lomize, T.M. Fong, D.H. Weinberg, R.P. Nargund, M.W. Mulholland, I. Grantz, H.I. Mosberg, Interactions of human melanocortin 4 receptors with nonpeptide and peptide agonists, *Biochemistry* 44 (2005) 11329–11341.
- [33] M. Rachel, H. El Mourabit, A. Buronfosse, A. Blondet, D. Naville, M. Begeot, A. Penhoat, Expression of the human melanocortin-2 receptor in different eukaryotic cells, *Peptides* 26 (2005) 1842–1847.
- [34] C.L. Reinick, L. Liang, J.K. Angleson, R.M. Dore, Functional expression of *Squalus acanthias* Melanocortin 5 Receptor in CHO Cells, ligand selectivity and interaction with MRAP, *Eur. J. Pharm.* 680 (2012) 1–7.
- [35] S. Roy, M. Rachel, N. Gallo-Payet, Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms  $\alpha$  and  $\beta$  in isogenic human embryonic kidney 293 cells, *Mol. Endocrinol.* 21 (2007) 1656–1669.
- [36] S. Roy, S.J. Roy, S. Pinard, L.-D. Tailler, M. Rachel, J.-I. Parent, N. Gallo-Payet, Mechanisms of melanocortin-2 receptor (MC2R) internalization and recycling in human embryonic kidney (HEK) cells: identification of key Ser/Thr (S/T) amino acids, *Mol. Endocrinol.* 25 (2011) 1961–1977.
- [37] S. Roy, S.J. Roy, S. Pinard, M.J. Agulleiro, J.M. Cerda-Reverter, J.-I. Parent, N. Gallo-Payet, The C-terminal domains of melanocortin-2 receptor (MC2R) accessory proteins (MRAP1) influence their localization and ACTH-induced cAMP production, *Gen. Comp. Endocrinol.* 176 (2012) 265–274.
- [38] R. Schwyzler, ACTH: a short introductory review, *Ann. N.Y. Acad. Sci.* 297 (1977) 3–26.
- [39] J.A. Sebag, P.M. Hinkle, Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers, *Proc. Natl. Acad. Sci. U S A* 104 (2007) 20244–20249.
- [40] J.A. Sebag, P.M. Hinkle, Regions of melanocortin (MC2) receptor accessory protein necessary for dual topology and MC2 receptor trafficking and signaling, *J. Biol. Chem.* 284 (2009) 610–618.
- [41] S. Seelig, B.D. Lindley, G. Sayers, A new approach to the structure-activity relationship for ACTH analogs using isolated adrenal cortex cells, in: J.G. Hardman, B.W. O'Malley (Eds.), *Methods in Enzymol.*, 39, Academic Press, New York, 1975, pp. 347–359.
- [42] T.R. Webb, J.L. Clark, Minireview: the melanocortin 2 receptor accessory proteins, *Mol. Endocrinol.* 24 (2010) 475–484.

## **APPENDIX 6**



## Minireview

## Evolution of melanocortin receptors in cartilaginous fish: Melanocortin receptors and the stress axis in elasmobranchs

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## ARTICLE INFO

Article history:  
Available online 31 August 2012

Keywords:  
MCR  
MC3R  
MC4R  
MC5R  
Cartilaginous fish  
Evolution

## ABSTRACT

There is general agreement that the presence of five melanocortin receptor genes in tetrapods is the result of two genome duplications that occurred prior to the emergence of the gnathostomes, and at least one local gene duplication that occurred early in the radiation of the ancestral gnathostomes. Hence, it is assumed that representatives from the extant classes of gnathostomes (i.e., Chondrichthyes, Actinopterygii, Sarcopterygii) should also have five paralogous melanocortin genes. Current studies on cartilaginous fishes indicate that while there is evidence for five paralogous melanocortin receptor genes in this class, to date all five paralogs have not been detected in the genome of a single species. This mini-review will discuss the ligand selectivity properties of the melanocortin-3 receptor of the elephant shark (subclass Holocephali) and the ligand selectivity properties of the melanocortin-3 receptor, melanocortin-4 receptor, and the melanocortin-5 receptor of the dogfish (subclass Elasmobranchii). The potential relationship of these melanocortin receptors to the hypothalamus/pituitary/interrenal axis will be discussed.

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## 1. Introduction

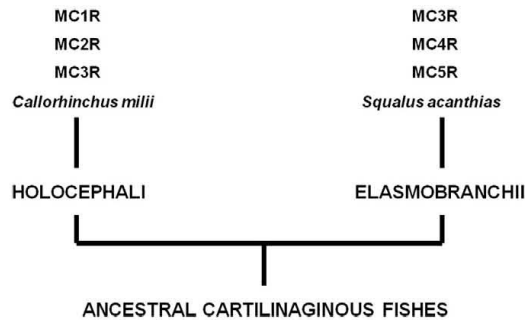
The presence of five melanocortin receptor-coding genes in the genomes of tetrapods coupled with the absence of melanocortin-related receptors in non-chordate metazoans [48] suggests that the evolution of this family of G-protein coupled receptors occurred during the evolution of phylum Chordata. As a result, the evolution of this gene family would have been influenced by the two generally accepted genome duplication events that occurred during the radiation of this phylum [34,38], and at least one localized gene duplication event [43]. Using the evolution of HOX genes as a model [25], the generally accepted scenario for the evolution of the melanocortin receptor gene family is that successive genome duplications in first the ancestral protochordates, and subsequently in the agnathan vertebrates, would have led to four paralogous melanocortin receptor-coding genes in the ancestral gnathostomes [48]. The presence of five paralogous melanocortin receptor-coding genes (i.e., *mc1r*, *mc2r*, *mc3r*, *mc4r*, *mc5r*) in the genomes of the teleost *Danio rerio* [32], and the tetrapods *Xenopus tropicalis* (<http://www.genome.jgi-psf.org/Xentr4/Xentr4.home.html>) and *Mus musculus* [10] provide support for the fifth melanocortin-coding gene resulting from a localized gene duplication [43]. Given these observations it was expected that five melanocortin receptors (MCRs) would be present in all of the extant classes of gnathostomes (i.e., Chondrichthyes, Actinopterygii, and Sacro-

pterygii; [36]. However, while studies on the cartilaginous fishes (class Chondrichthyes) have uncovered five paralogous *mcr* genes in representatives from the two subclasses of extant cartilaginous fishes (i.e., Holocephali and Elasmobranchii), all five *mcr* genes were not detected in the same species. Fig. 1 provides the current view of the phylogeny of the extant cartilaginous fishes, and the distribution of these *mcr* genes. A molecular phylogenetic analysis places the emergence of the ancestral cartilaginous fishes at approximately 445 MYA [42]. This analysis supports the monophyletic origin of subclass Holocephali and subclass Elasmobranchii. A subsequent evolutionary developmental biology study [18] and another molecular phylogenetic study [27] place the divergence of the two subclasses between 420 and 410 MYA. As shown in Fig. 1, an analysis of the genome database for the elephant shark, *Callorhynchus milii* (subclass Holocephali), revealed the partial amino acid sequence of a MC1R ortholog, and the full length sequences of orthologs of MC2R and MC3R, respectively [48]. However, within the genome of the dogfish, *Squalus acanthias* (subclass Elasmobranchii), only cDNAs corresponding to MC3R, MC4R, and MC5R were cloned and sequenced from this species [29,41]. It is possible that additional melanocortin receptor paralogs may be detected in the *C. milii* genome when this genome sequencing project is completed. In addition, sequencing of the *S. acanthias* genome is needed to determine whether additional melanocortin receptor paralogs are present in that genome.

Since the deduced amino acid sequence of the elephant shark MC1R ortholog is still incomplete, and the analysis of the elephant shark MC2R ortholog is in progress, this mini-review will focus on

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**Fig. 1.** Phylogeny of cartilaginous fish melanocortin receptors. In class Chondrichthyes, melanocortin receptors have been characterized from the dogfish, *Squalus acanthias* (subclass Elasmobranchii) [29,41], and the elephant shark, *Callorhynchus milii* (subclass Holocephali) [48]. Abbreviations: MC1R – melanocortin-1 receptor; MC2R – melanocortin-2 receptor; MC3R – melanocortin-3 receptor; MC4R – melanocortin-4 receptor; and MC5R – melanocortin-5 receptor.

the ligand selectivity of the MCR paralog common to both species, MC3R, and on the properties of the dogfish MC4R and MC5R orthologs. Observations on these melanocortin receptors will be used to consider the organization of the hypothalamus/pituitary/interrenal axis in elasmobranchs.

## 2. Elephant shark and dogfish MC3R orthologs

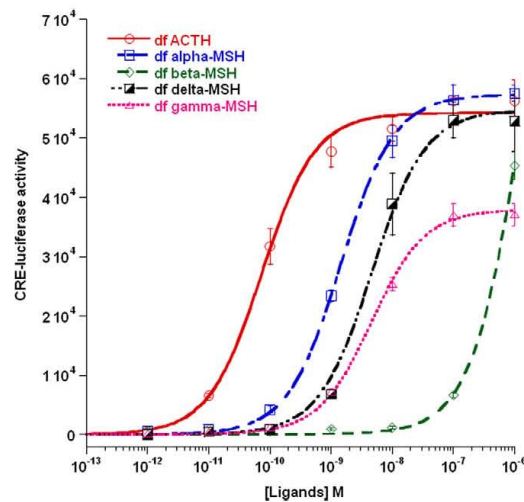
Fig. 2 presents an alignment of the amino acid sequences of elephant shark MC3R and dogfish MC3R. In these two receptor sequences, Intracellular Loop 1 (IC1), Transmembrane Domain 2 (TM2), and Intracellular Loop 2 (IC2) are identical, and the overall primary sequence identity of the two receptors is 83%. Dogfish MC3R has been functionally expressed in HEK-293 EBNA cells, and stimulation with dogfish ACTH(1–25) resulted in a robust production of cAMP in the transfected cells, whereas stimulation with dogfish  $\gamma$ -MSH was 100-fold weaker [20]. In this study stimulation of dogfish MC3R transfected cells by dogfish  $\alpha$ -MSH was not tested

in the functional expression assay. However, ligand binding assays were done for all of the dogfish melanocortins, and based on the binding assay analysis the order of ligand selectivity for dogfish MC3R is  $\text{ACTH}(1-25) = \alpha\text{-MSH} > \gamma\text{-MSH} > \delta\text{-MSH} \gg \beta\text{-MSH}$ . The efficacy of dogfish  $\beta$ -MSH on cAMP production by dogfish MC3R transfected HEK-293 cells was not tested, and binding studies indicated that dogfish MC3R was nearly 500-fold less sensitive to dogfish  $\beta$ -MSH binding as compared to the binding of dogfish  $\alpha$ -MSH [20]. Dogfish MC3R mRNA expression was detected in different regions of the dogfish brain including the hypothalamus, telencephalon, brain stem, and olfactory bulb [29], and this expression pattern is similar to observations on the mammalian central nervous system where MC3R has been implicated in the regulation of energy balance [8].

Ligand selectivity for elephant shark MC3R was based on the functional expression of this receptor in a heterologous assay using CHO cells, a derived mammalian immortalized cell line (Fig. 3). Dogfish ACTH(1–25) was clearly the preferred ligand ( $\text{EC}_{50} = 7.4 \pm 1.1 \times 10^{-11} \text{ M}$ ), whereas stimulation by dogfish  $\alpha$ -MSH ( $\text{EC}_{50} = 1.5 \pm 0.05 \times 10^{-9} \text{ M}$ ),  $\delta$ -MSH ( $\text{EC}_{50} = 4.7 \pm 0.6 \times 10^{-9} \text{ M}$ ), and  $\gamma$ -MSH ( $\text{EC}_{50} = 4.6 \pm 0.3 \times 10^{-9} \text{ M}$ ) was significantly weaker. Stimulation of elephant shark MC3R by dogfish  $\beta$ -MSH was only detected at the highest dose tested ( $10^{-6} \text{ M}$ ). Hence, elephant shark MC3R can be activated by either ACTH or the MSHs, but ACTH is clearly the more potent ligand ( $P < 0.0001$ ), and the order of ligand selectivity is  $\text{ACTH}(1-25) > \alpha\text{-MSH} > \delta\text{-MSH} = \gamma\text{-MSH} \gg \beta\text{-MSH}$ . Since it is expected that functional activation analyses should parallel ligand binding assays, it would appear that elephant shark MC3R has a clear preference for ACTH, whereas activation of dogfish MC3R should be achieved equally well using either dogfish ACTH(1–25) or  $\alpha$ -MSH. In this regard, the ligand selectivity of dogfish MC3R is similar to mammalian MC3R [16]. The weak binding affinity of dogfish  $\beta$ -MSH by dogfish MC3R, and the lack of stimulation by this melanocortin at physiological concentrations when tested with elephant shark MC3R are consistent with the observation that  $\beta$ -MSH is the most variable of the melanocortins detected in gnathostomes, and is most likely an evolutionary anachronism rather than a functional chemical signal at least in cartilaginous fishes [12]. MC3R mRNA expression has not been investigated in the central nervous system of the elephant

	N-terminal	[-----TM1-----]	IC1	
df	MD-----LNE <sup>*</sup> SVLNNRSSAGFCEQVPIKA <sup>*</sup> EVFLILGILSLENILVILSILKNNKLNHSPMY			71
es	MNSTHFLFDLQNGSGDLNKS <sup>*</sup> SILNRSNPGFCEQVPIKSEVFLTLGIISLENILVILAILKNNKLNHSPMY			
	[-----TM2-----]	EC1	[-----TM3-----]	
df	FFLCSLAVADMLVSVSNALETIVMAFLNNGYL <sup>*</sup> VANDQFIQMDNVFDSMICISLVASICNLLVIAIDRYIT			142
es	FFLCSLAVADMLVSVSNALETIVMALLNNGYL <sup>*</sup> VANDQFIQMDNVDSLICISLVASICNLLVIAIDRYIT			
	*	*	* *	
	IC2	[-----TM4-----]	EC2	[-----TM5-----]
df	IFYALRYHSIMTVKRALILDRSYLDCLYFCGIIFI <sup>*</sup> IYSDSKTAIICLITMFFTMLSSMTLYVHMFMLARL			213
es	IFYALRYHSIMTVKRALLLIIV <sup>*</sup> IACIFCGIIFI <sup>*</sup> IYNSKTVIICLITMFFTMLVLM <sup>*</sup> TTLYVHMFMLARL			
	IC3	[-----TM6-----]	EC3	[-----]
df	HIKRIATLPVNGMVRQRTCMKGAITITILLGIFIVCWAPFFLHLILIIISCPKNPYCICYTSHEFTYLILIM			284
es	HIKIAALPVDGIVRPRTCMKGAITITILLGIFII <sup>*</sup> CWAPFFLHLILIIISCPKNAYCICYTSHEFTYLILIM			
		*	*	*
	-TM7-----] C-terminal			
df	CNSVIDPIIYAFRSQEMRKTFKEIICCYCMNLNLRCK			
es	CNSVIDPMIYAFRSQEMRKTFKEIACCYGMNLNSRCKY			323

**Fig. 2.** Alignment of dogfish and elephant shark melanocortin-3 receptors. The amino acid sequences of dogfish MC3R (accession number: AAS66720.1) and elephant shark MC3R (accession number: FAA00705.1) were aligned as previously described [14]. One gap was inserted in this alignment. Positions that are identical are shaded. Underlined motifs indicate putative N-linked glycosylation sites. A (\*) indicates a residue that is found in the HPRW binding site of the HPRW melanocortin-4 receptor [39].



**Fig. 3.** Ligand selectivity of elephant shark melanocortin-3 receptor. CHO cells were transfected with the following plasmids: elephant shark MC3R (2  $\mu$ g) and cAMP reporter plasmid CRE-Luc (2  $\mu$ g) [9] using a Cell Line Nucleofector Kit (Amaxa, Inc.; <http://www.lonza.com>) as described in Reinick et al. [40]. The cells were plated on a white, 96-well plate at a density of  $1 \times 10^5$  cells per well. 48 h after transfection, cells were stimulated with the appropriate ligands in serum-free CHO media for four hours at 37 °C at concentrations ranging from  $10^{-6}$  M to  $10^{-12}$  M. After a 4 h incubation period, 100  $\mu$ l of Bright-Glo Luciferase assay reagent (Promega Inc., Madison, WI) was applied to each well and incubated at room temperature for 5 min. Luminescence was then measured with a Bio-Tek Synergy HT plate reader (Winooski, VT). The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: the dogfish melanocortins: ACTH(1–25),  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH [2].

shark; however, a similar expression pattern would be predicted. Since it appears that the elephant shark genome lacks a *mc4r* gene, the elephant shark MC3 receptor may be the most likely melanocortin receptor involved in the regulation of feeding behavior in this species.

### 3. Properties of dogfish MC4R

A MC4R ortholog was characterized from the genome of the dogfish [39]. This receptor has nearly 71% primary sequence identity with human MC4R. In addition, like the human ortholog, dogfish MC4R mRNA was detected in various regions of the central nervous system of the dogfish including the hypothalamus, telencephalon, brain stem, and olfactory bulb [39]; an expression pattern very similar to dogfish MC3R [29]. Based on these observations, it would be reasonable to assume that dogfish MC4R in the hypothalamus could be playing a role in the regulation of feeding behavior [8].

Functional expression of dogfish MC4R in HEK-293 EBNA also indicated that dogfish ACTH(1–25) was a more potent ligand than dogfish  $\gamma$ -MSH [20]. Once again dogfish  $\alpha$ -MSH was not tested in the functional assays done for this study. However, receptor binding assay analyses indicated that dogfish MC4R had 10-fold higher binding affinity for dogfish ACTH(1–25) as compared to dogfish  $\delta$ -MSH, and nearly 20-fold higher binding affinity as compared to dogfish  $\alpha$ -MSH [41]. Based on the binding assay analysis, the order of ligand selectivity for dogfish MC4R is ACTH(1–25) >  $\delta$ -MSH >  $\alpha$ -MSH >  $\gamma$ -MSH >  $\beta$ -MSH. This pattern of ligand selectivity is clearly different from the ligand selectivity of mammalian MC4R where it was found that  $\alpha$ -MSH binding affinity was equal to the binding

affinity of ACTH(1–24), and greater than the binding affinities of either  $\gamma$ -MSH or  $\beta$ -MSH [16].

### 4. Properties of dogfish MC5R

Although a cDNA encoding the deduced amino acid sequence of dogfish MC5R was characterized over 7 years ago [29], the ligand selectivity of this dogfish MCR was only determined recently [40]. In the former study attempts to functionally express a dogfish *mc5r* cDNA in HEK-293 cells were unsuccessful [29]. This observation was perplexing due to the fact that radioligand binding studies for other paralogous melanocortin receptor cDNAs from a variety of species have been successfully expressed in HEK-293 cells [19,28,31]. The exception to this generalization is the paralog MC2R. Attempts at expressing the mammalian ortholog of MC2R in cell lines not derived from the adrenal gland were unsuccessful. These observations led to the discovery of MRAP (melanocortin-2 receptor accessory protein) [35]. In many teleost and tetrapod species there are two *mrp* genes (1 and 2), and interaction between mammalian MC2R and the MRAP1 paralog not only facilitates the trafficking of mammalian MC2R to the plasma membrane but is also required for functional activation of the mammalian MC2R by ACTH [24,50]. Subsequent studies indicate that the functional expression of teleost or non-mammalian tetrapod MC2R orthologs in either HEK-293 or in CHO cells also require co-transfection with a *mrp1* cDNA [1,30].

Based on the preceding observations, it was possible that dogfish MC5R also required an accessory protein to facilitate trafficking to the plasma membrane and for functional activation. However, in a recent study it was found that dogfish MC5R could be visualized on the surface of CHO cells in the absence of the co-expression with mammalian or teleost MRAPs [40]. As expected, dogfish MC5R-transfected CHO cells could be activated by dogfish ACTH(1–25) as well as dogfish  $\alpha$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH. However, in this study it was observed that the order of ligand selectivity was ACTH(1–25) >  $\alpha$ -MSH >  $\gamma$ -MSH =  $\delta$ -MSH >  $\beta$ -MSH [38]. In fact, ACTH(1–25) was 10-fold more potent than  $\alpha$ -MSH and nearly 100 fold more potent than either  $\gamma$ -MSH or  $\delta$ -MSH. Once again  $\beta$ -MSH produced a minimal response above background at a concentration of  $10^{-6}$  M.

A RT-PCR analysis to study the distribution of dogfish MC5 receptors indicated that the expression of this receptor could be detected in the telencephalon and in the hypothalamus, but the *mc5r* expression was not apparent in peripheral organs [29]. However, a similar study on the distribution of *mc5r* expression in the shark *Heterodontus francisci* did detect *mc5r* expression in the head kidney [7]. This observation raises the possibility that MC5R plays a role in the HPI axis of elasmobranchs. This possibility will be discussed in the next section.

### 5. Ligand selectivity of cartilaginous fish MCRs

Focusing exclusively on the pharmacological properties of the cartilaginous fish MCR receptors considered in this review (i.e., elephant shark MC3R, dogfish, MC3R, MC4R, MC5R), a recurrent observation is the preference of these receptors for cartilaginous fish ACTH(1–25) as compared to any of the cartilaginous fish MSH sequences tested. This outcome is not unprecedented. The lamprey melanocortin-a receptor has higher affinity for mammalian ACTH(1–17) than any of the MSH sequences used in that study [21]. In addition, the rainbow trout MC4 receptor and the MC5R receptor had a higher affinity for ACTH(1–24) than any of the MSH-related ligands tested [19]. Although the sample size is small, it appears that ACTH could be the more potent ligand for agnathan, cartilaginous fish, and some teleost melanocortin receptors [20].





the rectal gland [4], and increased drinking rate [5,22]. Since  $1\alpha$ -OHB appears to function as both the mineralocorticoid and glucocorticoid steroid for cartilaginous fish, these observations raise the question of whether glucocorticoid effects occur as a result of angiotensin II stimulation of elasmobranch interrenal cells.

## 7. Conclusions

Although it is generally accepted that the cartilaginous fishes have a hypothalamus/pituitary/interrenal axis [17], unlike teleosts and tetrapods, there does not appear to be a melanocortin receptor on elasmobranch interrenal cells that can only be activated by ACTH [1,10]. To date all of the melanocortin receptors that have been functionally characterized from cartilaginous fishes can be activated by either ACTH or most of the MSH-related peptides derived from POMC. Hence, POMC-related melanocortins from either anterior pituitary cells or intermediate pituitary cells could activate peripheral target cells that express melanocortin receptors. Clearly additional work is needed on the mechanisms that regulate the secretion of melanocortins from elasmobranch anterior pituitary and intermediate pituitary cells, and the physiological functions of these melanocortins.

The receptor binding assays that have been performed on elasmobranch melanocortin receptors [20,29,40,41] and the functional expression assays that have been performed on elasmobranch [20] and holocephalan melanocortin receptors (Fig. 3) [40] expressed in heterologous mammalian cell lines indicate that ACTH is the preferred ligand for the activation of these melanocortin receptors. Based on the studies that have been done on lamprey [21] and cartilaginous fishes it would appear that ACTH may have been the ancestral ligand for the melanocortin receptors.

## Acknowledgment

This research was supported by NSF grant IOB 0516958 (R.M.D.).

## References

- [1] M.J. Agulleiro, S. Roy, E. Sanchez, P. Puchol, N. Gallo-Payet, J.M. Cerda-Reverter, Role of melanocortin receptor accessory proteins in the function of zebrafish melanocortin receptor type 2, *Mol. Cell. Endocrinol.* 320 (2010) 145–152.
- [2] Y. Amemiya, A. Takahashi, N. Suzuki, Y. Sasayama, H. Kawauchi, A newly characterized melanotropin in proopiomelanocortin in pituitaries of an Elasmobranch, *Squalus acanthias*, *Gen. Comp. Endocrinol.* 114 (1999) 387–395.
- [3] Y. Amemiya, A. Takahashi, N. Suzuki, Y. Sasayama, H. Kawauchi, Molecular cloning of proopiomelanocortin cDNA from an Elasmobranch, *Dasyatis akajei*, *Gen. Comp. Endocrinol.* 118 (2000) 105–112.
- [4] W.G. Anderson, M.C. Cerra, A. Wells, M.L. Tierney, B. Tota, Y. Takei, N. Hazon, Angiotensin and angiotensin receptors in cartilaginous fishes, *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* 128 (2001) 31–40.
- [5] W.G. Anderson, Y. Takei, N. Hazon, The dipsogenic effect of the rennin-angiotensin system in elasmobranch fish, *Gen. Comp. Endocrinol.* 124 (2001) 300–307.
- [6] B. Baker, The evolution of ACTH, MSH, and LPH-structure, function, and development, *Horm. Evol.* 2 (1980) 643–722.
- [7] A. Baron, K. Vee, J. Angleson, R.M. Does, Modeling the evolution of the MC2R and MC5R genes: Studies on the cartilaginous fish *Heterodontus francisci*, *Gen. Comp. Endocrinol.* 161 (2009) 13–19.
- [8] W. Chen, D.J. Marsh, M.E. Trumbauer, E.G. Frazier, X.M. Guan, H. Yu, C.I. Rosenthal, A. Vongs, Y. Feng, L. Cao, J.M. Metzger, A.M. Strack, R.E. Camacho, T.N. Mellin, C.N. Nunes, W. Min, J. Fisher, S. Gopal-Truter, D.E. MacIntyre, H.Y. Chen, L.H. Van der Poeg, Inactivation of the mouse melanocortin-3-receptor results in increased fat mass and reduced lean body mass, *Nat. Genet.* 26 (2000) 97–102.
- [9] O.G. Chepur, G.G. Holz, A novel cyclic adenosine monophosphate responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein coupled receptor drug discovery efforts, *J. Biomol. Screen.* 12 (2007) 740–746.
- [10] R.D. Cone, Studies on the physiological functions of the melanocortin system, *Endocr. Rev.* 27 (2006) 736–749.
- [11] R. deRoos, C.C. deRoos, Effects of mammalian ACTH on potential fuels and gluconeogenic substrates in the plasma of the spiny dogfish shark (*Squalus acanthias*), *Gen. Comp. Endocrinol.* 87 (1992) 149–158.
- [12] R.M. Does, A. Baron, Evolution of POMC: origin, phylogeny, posttranslational processing, and the melanocortins, *Ann. N.Y. Acad. Sci.* 1220 (2011) 34–48.
- [13] R.M. Does, S. Lecaude, Trends in the evolution of the proopiomelanocortin gene, *Gen. Comp. Endocrinol.* 142 (2005) 81–93.
- [14] R.M. Does, D.A. Rubin, T.W. Quinn, Mini-review: is it possible to construct phylogenetic trees using polypeptide hormone sequences, *Gen. Comp. Endocrinol.* 103 (1996) 1–12.
- [15] A.N. Evans, J.M. Rimoldi, R.S.V. Gadapalli, B.S. Nunez, Adaptation of a corticosterone ELISA to demonstrate sequence-specific effects of angiotensin II peptides and C-type natriuretic peptides on  $1\alpha$ -hydroxycorticosterone synthesis and steroidogenic mRNAs in the elasmobranch interrenal gland, *J. Steroid Biochem. Mol. Biol.* 120 (2010) 149–154.
- [16] I. Gantz, T.M. Fong, The melanocortin system, *Am. J. Physiol. – Endocrinol. Metab.* 284 (2003) E468–E474.
- [17] J. Gelsleichter, Hormonal regulation of elasmobranch physiology, in: J.C. Carrier, J.A. Musick, M.R. Heithaus (Eds.), *Biology of Sharks and their Relatives*, CRC Press, Boca Raton, FL, 2004, pp. 294–297.
- [18] J.A. Gillis, K.A. Rawlinson, J. Bell, W.S. Lyon, C.V.H. Baker, N.H. Shubin, Holocephalin embryos provide evidence for gill arch appendage reduction and opercular evolution in cartilaginous fishes, *Proc. Natl. Acad. Sci. USA* (2011) 1507–1512.
- [19] T. Haitina, J. Klovins, J. Anderson, R. Fredriksson, M.C. Lagerstrom, D. Larhammar, E.T. Larson, H.B. Schiöth, Cloning, tissue distribution, pharmacology and the three-dimensional modeling of melanocortin receptors 4 and 5 in rainbow trout suggest close evolutionary relationship of these subtypes, *Biochem. J.* 380 (2004) 475–486.
- [20] T. Haitina, A. Takahashi, L. Holmen, J. Enberg, H.B. Schiöth, Further evidence for ancient role of ACTH peptides at melanocortin (MC) receptors: pharmacology of dogfish and lamprey peptides at dogfish MC receptors, *Peptides* 28 (2007) 798–805.
- [21] T. Haitina, J. Klovins, A. Takahashi, M. Löwgren, A. Ringholm, J. Enberg, H. Kawauchi, E.T. Larson, R. Fredriksson, H.B. Schiöth, Functional characterization of two melanocortin (MC) receptors in lamprey showing orthology to the MC1 and MC4 receptor subtypes, *BMC Evol. Biol.* 7 (2007) 101–115.
- [22] N. Hazon, R.J. Balment, M. Perrott, L.B. O'Toole, The renin-angiotensin system and vascular and dipsogenic regulation in elasmobranchs, *Gen. Comp. Endocrinol.* 74 (1989) 230–236.
- [23] I.W. Henderson, J.A. Olivier, A. McKeever, N. Hazon, Physiological aspects of the renin-angiotensin system, in: G. Pelches, V.L. Frenyo (Eds.), *Advances in Physiological Sciences, Advances in Animal and Comparative Physiology*, vol. 2, Pergamon Press, London, 1981, pp. 355–363.
- [24] P.M. Hinkle, J.A. Sebag, Structure and function of the melanocortin 2 receptor accessory protein, *Mol. Cell. Endocrinol.* 300 (2009) 25–31.
- [25] P.W. Holland, J. Garcia-Fernandez, N.A. Williams, A. Sidow, Gene duplications and the origins of vertebrate development, *Development (Suppl.)* (1994) 125–133.
- [26] D.R. Idler, B. Truscott,  $1\alpha$ -Hydroxycorticosterone from cartilaginous fish: a new adrenal steroid in blood, *J. Fish. Res. Board Can.* 23 (1966) 615–619.
- [27] J.G. Inoue, M. Miya, K. Lam, B.-H. Tay, J.A. Danks, J. Bell, T.J. Walker, B. Venkatesh, Evolutionary origin and phylogeny of the modern holocephalans (Chondrichthyes; Chimaeriformes): a mitogenomic perspective, *Mol. Biol. Evol.* 27 (2010) 2576–2586.
- [28] J. Klovins, T. Haitina, D. Fridmanis, Z. Kilanova, I. Kapa, R. Fredriksson, N. Gallo-Payet, H.B. Schiöth, The melanocortin system in *Fugu*: determination of POMC/AGRP/MCR gene repertoire and synteny, as well as pharmacology and anatomical distribution of the MCRs, *Mol. Biol. Evol.* 21 (2004) 563–579.
- [29] J. Klovins, T. Haitina, A. Ringholm, M. Löwgren, D. Fridmanis, M. Slaidina, S. Stier, H.B. Schiöth, Cloning of two melanocortin (MC) receptors in spiny dogfish, *Eur. J. Biochem.* 271 (2004) 4320–4331.
- [30] L. Liang, J.A. Sebag, L. Egelston, M.N. Serasinghe, K. Vee, C. Reinick, J. Angleson, P.M. Hinkle, R.M. Does, Functional Expression of frog and rainbow trout melanocortin 2 receptors using heterologous MRAP1s, *Gen. Comp. Endocrinol.* 174 (2011) 5–14.
- [31] M.K. Ling, E. Hotta, Z. Kilanova, T. Haitina, A. Ringholm, L. Johansson, N. Gallo-Payet, S. Takeuchi, H.B. Schiöth, The melanocortin receptor subtypes in chicken have high preference to ACTH-derived peptides, *Br. J. Pharmacol.* 143 (2004) 626–637.
- [32] D.W. Logan, R.J. Bryson-Richarson, M.S. Taylor, P. Currie, I.J. Jackson, Sequence characterization of teleost fish melanocortin receptors, *Ann. N.Y. Acad. Sci.* 944 (2003) 319–330.
- [33] P.J. Lowry, H.P. Bennett, C. McMartin, The isolation and amino acid sequence of an adrenocorticotropin from the pars distalis and a corticotrophin-like intermediate-like peptide from the neurointermediate lobe of the pituitary of the dogfish *Squalus acanthias*, *Biochem. J.* 141 (1974) 427–437.
- [34] L.G. Lundin, Evolution of the vertebrate genome as reflected in paralogous chromosomal regions in man and the house mouse, *Genomics* 16 (1993) 1–19.
- [35] L.A. Metherell, J.P. Chappe, S. Cooray, A. David, C. Becker, F. Ruchendorf, D. Naville, M. Begeot, B. Khoo, P. Nurnberg, A. Huebner, M.E. Cheetham, A.J. Clark, Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2, *Nat. Genet.* 37 (2005) 166–170.
- [36] J.S. Nelson, *Fishes of the World*, third ed., John Wiley & Sons Press, New York, NY, 1994.

- [37] T.G. Nock, D. Chand, D.A. Lovejoy, Identification of members of the gonadotropin-releasing hormone (GnRH), Corticotropin-releasing factor (CRF) families in the genome of the holocephalon, *Callorhynchus milii* (elephant shark), Gen. Comp. Endocrinol. 171 (2011) 237–244.
- [38] S. Ohno, U. Wolf, N.B. Atkins, Evolution from fish to mammals by gene duplication, *Hereditas* 59 (1968) 169–187.
- [39] I.D. Pogozheva, B.-X. Chai, A.L. Lomize, T.M. Fong, D.H. Weinberg, R.P. Nargund, M.W. Mulholland, I. Grantz, H.I. Mosberg, Interactions of human melanocortin 4 receptors with nonpeptide and peptide agonists, *Biochemistry* 44 (2005) 11329–11341.
- [40] C.L. Reinick, L. Liang, J.K. Angleson, R.M. Does, Functional expression of *Squalus acanthias* Melanocortin 5 Receptor in CHO Cells, ligand selectivity and interaction with MRAP, *Eur. J. Pharm.* 680 (2012) 1–7.
- [41] A. Ringholm, J. Klovins, R. Fredriksson, N. Poliakova, E.T. Larson, J.P. Kukkonen, D. Larhammar, H.D. Schiöth, Presence of melanocortin (MC4) receptor in spiny dogfish suggests an ancient vertebrate origin of central melanocortin system, *Eur. J. Biochem.* 270 (2003) 213–221.
- [42] M. Robinson-Rechavi, B. Boussau, V. Laudet, Phylogenetic dating and characterization of gene duplication in vertebrates: the cartilaginous Fish Reference, *Mol. Biol. Evol.* 21 (2004) 580–586.
- [43] H.B. Schiöth, T. Haitina, M.K. Ling, A. Ringholm, R. Fredriksson, J.M. Cerda-Reverter, J. Klovins, Evolutionary conservation of the structural pharmacological and genomic characteristics of the melanocortin receptor subtypes, *Peptides* 26 (2005) 1886–1900.
- [44] A. Takahashi, T. Itoh, A. Nakanishi, Y. Amemiya, H. Ida, H. Meguro, H. Kawauchi, Molecular cloning of proopiomelanocortin cDNA in the ratfish a holocephalon, *Gen. Comp. Endocrinol.* 135 (2004) 159–165.
- [45] Y. Takei, Y. Hasegawa, T.X. Watanabe, K. Nakajima, N. Hazon, A novel angiotensin I isolated from an elasmobranch fish, *J. Endocrinol.* 139 (1993) 281–285.
- [46] M. Tierney, Y. Takei, N. Hazon, The presence of angiotensin II receptors in elasmobranchs, *Gen. Comp. Endocrinol.* 105 (1997) 9–17.
- [47] B. Uva, M.A. Masini, N. Hazon, L.B. O'Toole, I.W. Henderson, P. Ghiani, Renin and angiotensin converting enzyme in elasmobranchs, *Gen. Comp. Endocrinol.* 86 (1992) 407–412.
- [48] A. Vastermark, H.B. Schiöth, The early origin of melanocortin receptors, agouti-related peptide, agouti signaling peptide, and melanocortin receptor-accessory proteins, with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus, *Eur. J. Pharmacol.* (2011), <http://dx.doi.org/10.1016/j.ejphar.2010.10.106>.
- [49] D. Waugh, G. Anderson, K.J. Armour, R.J. Balmont, N. Hazon, J.M. Conlon, A peptide from the caudal neurosecretory system of the dogfish, *Scyliorhinus canicula*, that is structurally related to urotensin I, *Gen. Comp. Endocrinol.* (1995) 333–339.
- [50] T.R. Webb, A.J. Clark, Minireview: the melanocortin 2 receptor accessory proteins, *Mol. Endocrinol.* 24 (2010) 1–10.